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VOLUME 37 NUMBER 3

JOURNAL OF BACTERIOLOGY

OFFICIAL ORGAN OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

MARCH, 1939

EDITOR-IN-CHIEF
C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our vision—PASTEUR

Published Monthly by THE WILLIAMS & WILKINS COMPANY
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Entered as second-class matter April 17, 1916 at the postoffice at Baltimore, Maryland, under the Act of March 3, 1879.
Subscription price $5.00 per volume. Single numbers, $1.00.

THE RELATION OF AEROBIOSIS TO THE FERMENTATION OF MANNITOL BY STAPHYLOCOCCI

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Received for publication August 5, 1938

While studying the fermentative reactions of a number of strains of *Staphylococcus aureus*, it was noticed that the mannitol tubes differed in appearance from those of the fermentable carbohydrates glucose, lactose, and sucrose. In the mannitol tubes (1 per cent mannitol semi-solid agar with Andrade indicator) only the top of the medium to a depth of $\frac{1}{8}$–$\frac{1}{4}$ inch was pink after 24–48 hours incubation, while in the other tubes the medium was uniformly pink. This observation led to the study of the fermentation of mannitol by staphylococci from various sources with special reference to their oxygen requirements. Six strains of mannitol-fermenting streptococci were tested in comparison.

The fermentation of mannitol by staphylococci has been studied by a number of investigators, Hine (1922), Dudgeon and Simpson (1928), Julianelle (1937), Thompson and Khorazo (1937), and others, and their conclusions are in close agreement that the pathogenic strains, (*Staphylococcus aureus* and *Staphylococcus albus*), are, in the main, mannitol-positive, while the nonpathogenic ones (usually *S. albus*) are, with few exceptions, mannitol-negative. With regard to the oxygen requirements for the growth of staphylococci, the test books list these organisms as facultative anaerobes which grow best in the presence of oxygen. Fildes and his co-workers (1936) found that a synthetic nutrient mixture that supported aerobic growth of staphylococci was inadequate for anaerobic growth without the addition of three other substances, namely pyruvic acid, CO_2, and a

"staph factor" which was shown later by Knight (1937) to consist of nicotinamide and aneurin (Vitamin B_1).

METHODS

Solutions of the test substances glucose, lactose, sucrose, and mannitol (Pfanstiehl), sterilized by Berkefeld filtration, were added in 1 per cent amounts to the basic media, 1 per cent Difco Bacto peptone, pH 7.0, and semi-solid agar containing 0.5 per cent peptone, pH 7.1. Andrade and brom-cresol-purple were used as indicators. After inoculation of a small loop of 18-hour slant growth, free access of oxygen was excluded from certain tubes by a layer of sterile vaseline on top of the medium.

SOURCE OF CULTURES

Six of the staphylococcus cultures from boils, all from normal throats, normal skin, and cream filling were freshly isolated and tested within two weeks. The rest were stock cultures isolated within five years. All colonies fished from each individual source were identical by mannitol fermentation.

Only cultures of staphylococci which were found to ferment mannitol aerobically are included in this study. Other cultures were tested which failed to ferment under both aerobic and anaerobic conditions; they included 4 *S. aureus* strains (normal throat and normal skin) and 10 *S. albus* (normal throat, normal skin and infected sebaceous gland).

The results of the fermentation tests of 25 *Staphylococcus aureus*, 5 *Staphylococcus albus*, and 6 *Streptococcus* cultures in mannitol, glucose, lactose, and sucrose mediums in test tubes with and without a layer of vaseline are summarized in the table. All of the staphylococcus cultures fermented mannitol within 48 hours when grown aerobically at 37°C. The semi-solid medium showed the indicator change only at the top of the medium during 24–48 hours incubation except for two cultures, H and C, which were slightly acid in the butt as well; by the third day of incubation all tubes were uniformly acid. When grown anaerobically, 28 of the 30 cultures were negative up to the 5th day, but after 6–14 days small and irregular amounts of indicator change were

noted. The 6 cultures of *Streptococcus* fermented mannitol within 24 hours under both aerobic and anaerobic conditions.

The carbohydrates glucose, lactose, and sucrose were fermented promptly by all of the cultures of *Staphylococcus* and *Streptococcus* when grown with and without vaseline seal.

Other basic media, namely extract broth and 10 per cent horse-serum water containing mannitol were tested with a limited

TABLE 1

Fermentation of mannitol, glucose, lactose, and sucrose by staphylococci and streptococci when tested aerobically and anaerobically (vaseline seal)

ORGANISM	SUB-GROUP	SOURCE	NUMBER	MANNITOL			GLUCOSE, LACT., SUCROSE	
				Aerobic	Anaerobic		Aerobic	Anaerobic
				48 hours	48 hours	5 days	48 hours	48 hours
Staphylococcus	*Aureus*	Boils, etc.	16	+	−	−	+	+
		Boil (H)	1	+	±	+	+	+
		Blood	1	+	−	−	+	+
		Skin (normal)	2	+	−	−	+	+
		Throat (normal)	2	+	−	−	+	+
		Throat (normal) (C)	1	+	±	±	+	+
		Cream filling	2	+	−	−	+	+
	Albus	Boil	1	+	−	−	+	+
		Skin (normal)	1	+	−	−	+	+
		Throat (normal)	3	±−+	−	−	+	+
Streptococcus	*Alpha*	Feces	2	+	+	+	+	+
		Milk	2	+	+	+	+	+
	Beta	Milk	2	+	+	+	+	+

Explanation of symbols: +, acid; ±, weakly acid; − no change in indicator.

number of cultures. The results coincided with those obtained in the peptone and semi-solid media.

More concise data on the relation of free access of oxygen to the fermentation of mannitol by staphylococci resulted from a comparison of the pH readings on a culture incubated in a shallow layer of mannitol-peptone medium in a flask and in test tubes, with and without vaseline seal. pH readings were made by the

colorimetric method. After 24 hours the pH of the growth in the flask was 4.9, in the open tube 6.0, and in the closed tube 7.0; after 48 hours the readings of the growth in the respective environments were 4.5, 5.2, 7.0; and after 5 days 4.5, 4.5, 6.8. Four additional cultures were grown under vaseline seal in duplicate for 5 days. The pH readings ranged from 6.3 to 6.9. Duplicate tubes, in some instances, gave different pH readings, which were usually associated with slight differences in cloudiness.

GROWTH OF STAPHYLOCOCCUS UNDER ANAEROBIC CONDITIONS

The small changes in pH reading and the occasional slight increased cloudiness after 5 days incubation under vaseline seal would indicate that the organisms had grown to some extent. More definite proof was obtained when serial dilutions of culture in semi-solid mannitol-agar were incubated with and without vaseline seal. Observations made after 24 hours incubation showed that, in the highest culture dilution, approximately the same number of colonies had developed in the aerobic and anaerobic tubes, but the colonies in the closed tube were much smaller than in the aerobic one and they did not increase in size upon incubation for 5 days.

FERMENTATION OF GLYCEROL AND SORBITOL

Because of the observed difference in the fermentative reactions of staphylococci in the alcohol mannitol as compared with the carbohydrates glucose, lactose, and sucrose, it seemed of interest to test reactions in the alcohols glycerol and sorbitol. The result of this test showed that 8 mannitol-positive cultures, including strain H, fermented glycerol aerobically in 48 hours to 5 days but failed to ferment within 14 days when incubated under vaseline seal. A mannitol-negative strain of *Staphylococcus* was also negative in glycerol.

Sorbitol was not fermented by 10 cultures of *Staphylococcus* incubated aerobically for 12 days. Ten per cent horse-serum water with 1 per cent sorbitol was used in addition to the peptone and semi-solid mediums. Sorbitol was fermented within 24 hours by 2 cultures of beta-hemolytic streptococcus of Lancefield (1933) group C when grown aerobically and under vaseline seal.

DISCUSSION

The results of these experiments, which show distinct differences in the anaerobic actions of staphylococci on mannitol and glycerol on the one hand and on glucose, lactose, and sucrose on the other, but not in the aerobic actions on these same test substances, are presented without attempting to enter further into the chemical, metabolic, or enzymatic factors involved. A possible explanation of these findings may be based on difference in chemical composition, the former compounds being alcohols and the latter carbohydrates; and the probability that under anaerobic conditions the alcohols are not utilized as readily as the carbohydrates by an organism which grows feebly anaerobically. The studies of Gladstone, Fildes, and Richardson (1935) may have some bearing on this work. They found that "in an anaeroboid CO_2-free atmosphere, *Staphylococcus aureus* did not grow in 45 hours but that if glucose or lactate were present growth occurred in 24 hours."

SUMMARY

Twenty-eight cultures of *Staphylococcus* (23 *S. aureus* and 5 *S. albus*) which fermented mannitol in 24–48 hours aerobically, when tested under vaseline seal showed no change of indicator after 5 days incubation, but small and irregular amounts on longer incubation. pH readings on a limited number of cultures, incubated for 5 days under vaseline seal, ranged from 6.3 to 6.9. Two cultures of *Staphylococcus aureus* showed slight to moderate fermentation of mannitol after 48 hours incubation under vaseline seal.

Eight cultures of mannitol-fermenting staphylococci fermented glycerol aerobically in from 48 hours to 5 days, but failed to ferment when grown anaerobically for 14 days.

The fermentation of glucose, lactose, and sucrose by the 30 cultures of *Staphylococcus* took place within 48 hours aerobically and under vaseline seal.

Unlike the staphylococci, 6 *Streptococcus* cultures (4 alpha and 2 beta) fermented mannitol as well as glucose, lactose, and sucrose within 48 hours whether grown under aerobic or anaerobic conditions.

The marked inhibition or lack of fermentation of mannitol anaerobically by staphylococci is dependent, to some extent at least, upon the limited growth activity in an anaerobic environment.

It is suggested as a possible explanation that staphylococci cannot utilize the alcohols mannitol and glycerol as readily as the carbohydrates glucose, lactose, or sucrose in order to obtain substances essential for vigorous growth under anaerobic conditions.

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A SKUNK-LIKE ODOR OF BACTERIAL ORIGIN IN BUTTER[1]

T. J. CLAYDON AND B. W. HAMMER

Received for publication August 8, 1938

Butter is subject to various types of microbiologic deterioration. Some of these are encountered frequently, others quite infrequently, in the butter industry. Occasionally, a defect is noted that has not been observed previously in a given butter-producing section, or at least has not been differentiated from other objectionable conditions.

The work herein reported deals with an unusual butter defect in which the product developed a skunk-like odor. The organism responsible for the condition was isolated.

EXPERIMENTAL

General studies

Three samples of commercial butter, from a series being held under observation at 21°C. to test the keeping qualities, developed a skunk-like odor, sample 1 after 2 days and samples 2 and 3 after 4 days. The defect was most conspicuous in sample 1. The samples were unsalted and originally scored 93. They were from the same plant; another sample from the plant did not develop the defect and later samples also failed to do so.

Approximately 10-gram portions from samples 1 and 2 were inoculated separately into pasteurized sweet cream and the cream churned. The resulting lots of unsalted butter were divided and stored at 21° and about 5°C. In 2 days at 21°C. the samples developed the odor of the original butter, sample 1 causing the defect to a much greater extent than sample 2. In 6 days the

[1] Journal Paper J-571 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project No. 119.

samples stored at about 5°C. developed the off odor, sample 1 again exhibiting a much more conspicuous defect than sample 2.

The procedure was repeated, using the defective experimental butter for inoculation of the cream. The defect developed in the lot representing sample 1 but not in the other. From the former sample the defect was carried through five successive churnings by inoculating cream with the defective butter made just previously; additional churnings were not attempted.

When the first experimental churnings were made the original butter was examined bacteriologically. The usual plating method was employed and also the method of Long and Hammer (1938) in which tiny portions (approximately 1/20,000 gram) are smeared on sections of agar plates. Beef-infusion agar, with skim milk and fat emulsion added just before pouring, was used. The numbers of bacteria, as shown by plating, were as follows:

Bacteria per ml.

Sample 1:		Sample 2:	
Total	31,000,000	Total	185,000,000
Proteolytic	21,000,000	Proteolytic	35,000,000
Lipolytic	9,000,000	Lipolytic	27,000,000

The floras of the two samples were similar and comprised three main colony types—green fluorescent, greyish white and bluish transparent. In general, the types of colonies on smeared plates were the same as on poured plates.

Pure cultures of the conspicuous colony types inoculated into pasteurized sweet cream failed to produce the skunk-like odor in unsalted butter made from the cream. Various combinations of these types, as well as cultures of less common types, also gave negative results.

The first samples of experimental butter were examined bacteriologically in the same manner as the original butter. The plate counts on the samples were as follows:

Bacteria per ml.

Sample 1:		Sample 2:	
Total	770,000,000	Total	620,000,000
Proteolytic	80,000,000	Proteolytic	110,000,000
Lipolytic	11,000,000	Lipolytic	14,000,000

In general, the colony types were the same as with the original butter. Pure cultures obtained from the defective experimental butter, as well as various combinations of these, failed to reproduce the defect.

Since the reproduction of the unusual odor in series indicated that the defect was due to an organism, attempts were made to increase the proportion of the causative type by culturing the butter in litmus milk at about 5°C. These cultures developed the skunk-like odor in 4 to 5 days. Successive transfers from them reproduced the odor in litmus milk. Unsalted butter made by inoculating pasteurized sweet cream with the cultures also developed the skunk-like odor. On streaking the litmus milk cultures, or the butter, on beef-infusion agar the same general flora developed as from the original butter.

Since the skunk-like odor suggested the breakdown of sulfur compounds, nutrient broth containing 0.02 per cent cystine was used in an attempt to increase the number of odor-producing organisms. Defective butter inoculated into this medium produced a strong hydrogen sulfide odor in 1 or 2 days. However, the same result was obtained with a sample of normal product.

Butter having the skunk-like odor was plated on beef-infusion agar containing lead acetate and cystine and colonies producing blackened areas were picked into litmus milk. The organisms thus obtained did not give the odor in experimental butter made from pasteurized sweet cream into which they had been inoculated.

At this point, it appeared that the causative organism was not developing on beef-infusion agar and additional media were employed. With each medium the conspicuous colony types were picked and tested in the usual way. One of the media consisted of 4 per cent casein and 1.5 per cent agar in water; the casein was dissolved in a small amount of water by means of sodium hydroxide and the agar in the remainder of the water by boiling, after which the two were mixed and the pH adjusted to approximately 7.0. From plates of this medium streaked with defective butter and incubated at 21°C. a number of colonies were picked that gave the skunk-like odor in litmus milk. The

colonies were rather characteristic in appearance and constituted from 5 to 10 per cent of the total number. When litmus milk cultures from these colonies were inoculated into pasteurized sweet cream and the cream churned, the resulting unsalted butter developed an odor like that of the original butter. From such butter the organism inoculated into the cream was easily recovered by smearing on the casein medium.

After the isolation of the organism from casein agar plates, beef-infusion agar plates smeared at the same time were examined for its presence. No colonies were observed on plates incubated at 21°C. but later observations showed small numbers on plates incubated at about 5°C. Subsequent trials indicated that the organism grew well on beef-infusion agar at 21°C. It was probably missed originally because of the relatively small numbers of colonies present, the percentage of the total colonies made up of the causative organism being smaller on beef-infusion agar than on casein agar as a result of the suitability of the former for growth of many species. The organism also grew on various other agar media but not on tomato agar. In some of the later trials beef-infusion agar was used for isolation.

The organism was studied in detail and was considered to be an undescribed species. The name *Pseudomonas mephitica* is suggested for it. The following description is based on a number of cultures isolated from the butter studied.

Description of Pseudomonas mephitica[2]

Morphology (at 21°C.). Form and size—Rods; in young beef-infusion agar slope cultures the cells varied from 0.5 to 1.0 by 1.5 to 14.0 microns and averaged about 0.6 by 3.8 microns. Older cultures generally had more of the longer cells.

Arrangement—Singly, pairs and chains, in preparations from agar or milk.

Motility—Actively motile with one polar flagellum which sometimes did not appear to be exactly at the end of the cell.

Staining reactions—Stained readily with the usual stains; gram-negative; granules generally present.

[2] The description was checked by Dr. H. F. Long.

Spore formation—Spores not observed; the organism was easily destroyed by heat.

Cultural characteristics (at 21°C.). Agar slope—Growth on beef-infusion agar slopes was echinulate, greyish white and wrinkled. After 1 or 2 days the slopes had a skunk-like odor.

Agar stab—In 2 to 3 days beef-infusion agar stabs showed a greyish white, wrinkled surface growth; along the line of inoculation growth was very meager.

Agar colony—On beef-infusion agar, colonies were evident in 1 to 2 days; well developed surface colonies were convex, circular, about 3 mm. in diameter, shiny, greyish white, with an entire edge and a fine granular or feathered appearance. Older colonies were about 5 mm. in diameter, convex and slightly wrinkled at the center. Subsurface colonies were generally elliptical. All colonies were of a doughy consistency which permitted them to be picked rather completely from the agar.

Gelatin stab—Growth was essentially the same as in an agar stab and liquefaction, evident after several days, progressed slowly and was crateriform changing to stratiform.

Bouillons—Bouillons showed a turbidity, which gradually extended downward and settled as sediment, and a white, easily broken pellicle.

Dunham's solution—Growth was the same as in bouillons.

Uschinsky's solution—Slight turbidity developed and settled as a white sediment.

Potato—Growth on potato was echinulate, shiny and slightly brown.

Litmus milk—A skunk-like odor developed in 1 to 2 days, a greyish blue ring at the surface in about 3 days, and a definite alkalinity in 7 to 10 days. Reduction began at the bottom of the culture in about 7 days and was generally complete in 14 days. At this time the litmus milk was slightly proteolyzed and somewhat viscous; certain strains showed a slight viscosity earlier.

Plain milk—Except for reduction of litmus, the action in plain milk was the same as in litmus milk.

Biochemical characteristics (at 21°C.). Gas production—None.

Hydrogen sulfide—Not produced.

Indole—Not produced.

Acetylmethylcarbinol from glucose—Not produced.

Methyl-red reaction—Negative.

Nitrates—Reduced to nitrites.

Ammonia—Produced in nitrate broth and peptone broth.

Reaction change—Acid was not produced from arabinose, dextrin, galactose, glycerol, lactose, mannitol, raffinose or salicin. It was slowly produced from glucose, levulose, maltose and sucrose, with some tendency to reversal of reaction. Acid production was especially slow from glucose.

Natural fats—Not hydrolyzed.

Growth conditions. Oxygen relationships—Facultative.

Growth temperatures—Grew well at 21°C., considerably slower at 5°C., slight growth at 30°C., but none at 37°C.

Effects of various factors on development of the skunk-like odor in butter by pure cultures

In unsalted experimental butter, made by inoculating a pure culture of the organism into pasteurized sweet cream, the skunk-like odor commonly developed in 2 days at 21°C. and in about 6 days at approximately 5°C. Repeated trials gave consistent results. The effects of various factors on the development of the defect were then considered.

Effect of pH of the cream. A series of churnings was made from six portions of pasteurized sweet cream adjusted to pH values ranging from 5.6 to 7.3 at the time of inoculating the culture into the cream. After incubating overnight at about 10°C., the cream was churned. In 2 days the defect was present in all six samples of butter at 21°C. The odor was least conspicuous at the lowest pH. In another series of three portions of cream adjusted to pH values of 5.0, 6.5 and 7.5, the defect developed in all the butter at 21°C. but was somewhat less pronounced than in the first series. Again, the odor was least conspicuous at the lowest pH.

Effect of salting and working. The effect of salting and working butter on the development of the defect at 21°C. is illustrated by the following summary. The salt percentages are based on the salt added rather than on analyses of the butter.

The data, confirmed in other trials, show that the working the salted butter received was a factor in controlling the defect. While salt tended to inhibit development of the odor, it was not completely effective unless combined with thorough working.

SALT ADDED	WORKING OF BUTTER	DEFECT DEVELOPED
None	Moderate	Pronounced after 2 days
1 per cent	Slight	Definite after 2 days
	Moderate	Slight after 3 days
	Thorough	Questionable after 6 days
2 per cent	Slight	Definite after 2 days
	Moderate	Questionable after 3 days
	Thorough	None after 6 days

Effect of contamination of the wash water. The defect was produced in unsalted experimental butter when small amounts of pure culture were added to the water used to wash the butter. It usually was evident in 2 to 3 days at 21°C.

Relationship to other defects

Certain creameries in the southwest have reported an odor in butter which is suggestive of a skunk odor. It is often attributed to one or more plants consumed by the cows in the pastures. Over a period of about 1 year, each of three samples of such butter was inoculated into pasteurized cream and the cream churned in order to determine whether the defect could be reproduced. In each case the original butter was salted; the skunk odor was definite but differed somewhat from the defect produced by the culture isolated. None of the experimental butter, which was unsalted, developed the odor of the original butter.

DISCUSSION

The development of a skunk odor in butter through the action of a bacterial species illustrates the unusual defects caused in this product by microorganisms. The list of such defects will undoubtedly be extended as improved manufacturing procedures limit the total number of organisms in butter and still occasionally permit one or more particularly harmful species to be present in sufficient numbers to cause conspicuous changes.

The defect studied, while definitely suggesting a skunk odor, varied somewhat from one experimental lot of butter to another as a result of an increase and then a decrease in intensity. At

certain stages the odor was comparable to that of decomposing cabbage. The similarity of the defect to an off flavor in butter that is generally regarded as due to feed is of importance in showing that organisms can produce defects suggestive of those caused in other ways.

Since *Pseudomonas mephitica* grew at relatively low temperatures, over the usual pH range used in buttermaking, and in salted butter that was only moderately worked as well as in unsalted butter, it undoubtedly would be of significance commercially when considerable numbers are added to pasteurized cream intended for butter, or to the butter itself. The low thermal resistance of the organism indicates that proper pasteurization and sanitary methods in the plant will control the defect it causes.

SUMMARY

Three samples of high quality, unsalted butter from one plant developed a skunk-like odor on holding at 21°C. to test the keeping qualities. When the defective butter was inoculated into pasteurized sweet cream and the cream churned, the defect was reproduced. The causative organism was isolated and described; the name *Pseudomonas mephitica* is suggested for it. On inoculating the pasteurized cream used for churning, the defect developed in unsalted butter made from cream having pH values from 5.0 to 7.5. Salt had an inhibitory effect on the production of the defect but thorough working of the salted butter was necessary to prevent its appearance. The skunk-like odor was produced in unsalted butter by inoculating small amounts of culture into water used to wash the butter.

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GROWTH FACTORS FOR BACTERIA

VII. Nutrient Requirements of Certain Butyl-Alcohol-Producing Bacteria[1]

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Received for publication August 10, 1938

With the exception of *Clostridium pasteurianum* none of the butyl-alcohol-producing bacteria have been satisfactorily cultivated in synthetic media. Usually these organisms are cultured in corn mash, in yeast-water glucose, or in an inorganic salts-peptone-glucose medium (McCoy *et al.*, 1930). Apparently, there is in corn, yeast and peptone some compound or compounds essential for the growth of these organisms.

Tatum, Peterson and Fred (1935) found l-asparagine or related dicarboxylic acids necessary for a normal fermentation by *Clostridium butylicum* in corn mash.

Weizmann and Rosenfeld (1937) obtained a normal fermentation of carbohydrate by *Clostridium acetobutylicum* in a medium which included substances of known composition and a growth-promoting substance contained in a dialysate of autolyzed yeast.

Normal yields of solvents were obtained by Brown, Wood and Werkman (1938) by growing butyl-alcohol-producing bacteria in a medium of hydrolyzed casein, ammonium sulfate, tryptophane, glucose, inorganic salts and an acidic ether-soluble extract of Difco yeast extract.

This paper deals with the occurrence and partial purification of an accessory substance which permits growth of *C. butylicum* and *C. acetobutylicum* in a medium containing inorganic salts, asparagine and glucose.

[1] This work was aided by a grant from the Wisconsin Alumni Research Foundation. Published with the approval of the Director of the University Agricultural Experiment Station.

EXPERIMENTAL

Cultures and methods

The bacteria employed were *C. butylicum* strain 21 (Langlykke, Peterson and Fred, 1937), a butyl-alcohol isopropyl-alcohol-forming culture; and *C. acetobutylicum* Weizmann (McCoy, Fred, Peterson and Hastings, 1926), a butyl-alcohol acetone-producing culture. The same spore stock in soil was used throughout the study.

The basal medium contained 2 per cent glucose, 0.1 per cent asparagine, acid hydrolyzed casein, Speakman's inorganic salts,[2] reduced iron and distilled water. For *C. acetobutylicum*, 0.25 per cent hydrolyzed casein was used. Addition of tryptophane to the hydrolyzed casein did not improve fermentation. For *C. butylicum* 0.05 per cent casein was used at first, but later it was omitted and a purely synthetic base medium employed. Only slight growth occurred in the unsupplemented basal medium. The relative amounts of accessory growth factor in different source materials were determined in the early experiments by noting the comparative rates of fermentation of glucose when such materials were added to the basal medium. Each substance was tested in several concentrations, the total volume of medium in each case being 35 ml. Since a small amount of growth always occurred in the unsupplemented basal medium, each time assays were made the basal medium alone was assayed.

The inocula were prepared as follows. Each time assays were made, a fresh culture was started from the spore stock by inoculating into a medium of the same composition as that described above except that hydrolyzed casein was replaced by Bacto-Peptone (0.5 per cent for *C. acetobutylicum*, 0.25 per cent for *C. butylicum*). Each tube of medium was placed in boiling water for five minutes prior to inoculation and again for two minutes after inoculation. All cultures were incubated at 37°C. in anaerobic oat jars. Twenty-four hours later these cultures

[2] Speakman's salts: K_2HPO_4, 0.5 gram; KH_2PO_4, 0.5 gram; $MgSO_4 \cdot 7H_2O$, 0.2 gram; NaCl, 0.01 gram; $FeSO_4 \cdot 7H_2O$, 0.01 gram; and $MnSO_4 \cdot 3H_2O$, 0.01 gram per liter.

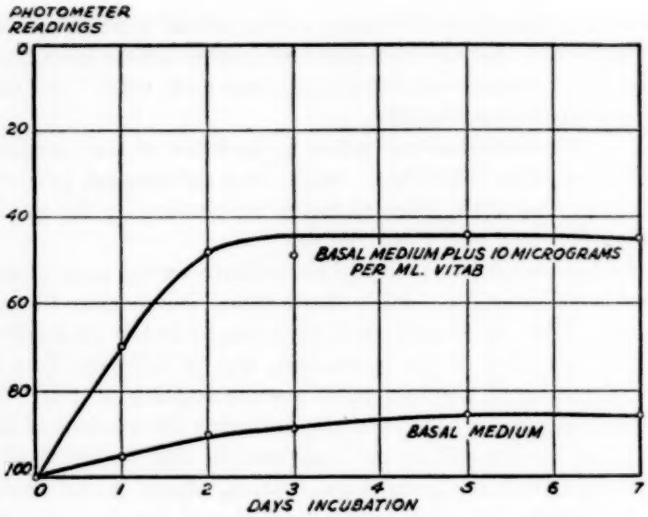

FIG. 1. DEVELOPMENT OF TURBIDITY IN CULTURES OF C. BUTYLICUM

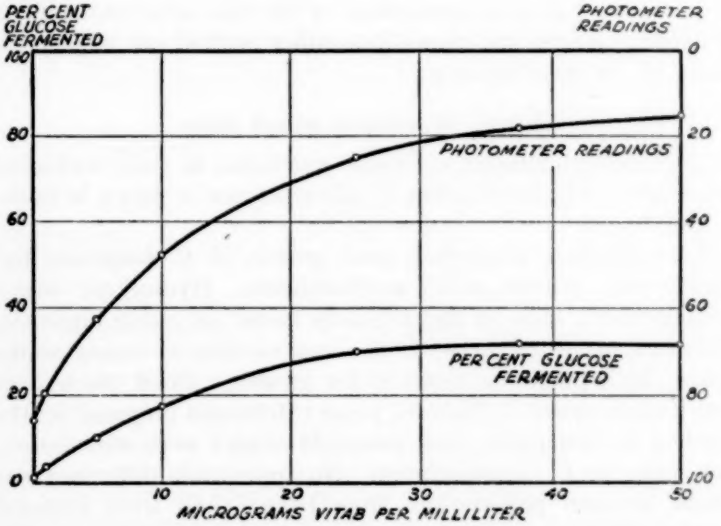

FIG. 2. COMPARISON OF RATE OF FERMENTATION AND CULTURE TURBIDITY ASSAYS ON CULTURES OF C. BUTYLICUM

were used to inoculate the assay tubes, which were also heated in boiling water for five minutes and cooled before inoculation. 0.35 ml. of inoculum was introduced into each tube. All tubes were incubated anaerobically.

Glucose fermentation was taken as an index of the amount of growth-promoting substance. Sugar was determined just after inoculation, and again after 48 hours' incubation by the method of Stiles, Peterson and Fred (1926).

In the later work, turbidity of the culture served as a criterion of growth stimulation. After three days' incubation the turbidity was determined with an Evelyn photoelectric photometer. The light intensity of the instrument was so adjusted that the uninoculated basal medium gave a scale reading of 100. The scale reading given by each culture indicated the amount of light transmitted by the culture as a per cent of that transmitted by the uninoculated basal medium. Thus, there is an inverse relation between the photometer reading and the degree of turbidity of the culture.

The maximum turbidity was reached in two to three days (fig. 1). Figure 2 gives a comparison of the two assay methods on the same cultures and shows that either method can be used for assay of the growth factor.

Sources of accessory growth factor

A growth-stimulating substance was found in many biological materials. The distribution of this substance is shown in tables 1 and 2.

Bacto-Peptone supported good growth of *C. butylicum* but rather poor growth of *C. acetobutylicum*. Hydrolyzed casein contained but little of the accessory factor for either organism. This fact suggested its use in the base medium to supply amino acids which might be essential for growth. Dried whole liver was a good source. Dialyzed yeast autolysates prepared by the method of Weizmann and Rosenfeld (1937) were stimulatory, especially for *C. acetobutylicum*. No appreciable difference was found between preparations from bakers' and from brewers' yeast. Alcoholic extracts of yellow corn were high in activity. Extractions with other solvents yielded less active preparations

and in most cases the amount of extract also was less. Alcoholic extracts of wheat bran were somewhat less active for *C. butylicum*

TABLE 1

Effects of various supplements on rate of fermentation of glucose by C. butylicum

SUPPLEMENT	ORIGINAL MATERIAL IN SUPPLEMENT	SUPPLEMENT ADDED (MICROGRAMS PER ML.) AND GLUCOSE FERMENTED (PER CENT)						
		0*	1	10	50	100	250	500
	per cent							
Bacto peptone	100	13.3				38.8	47.5	55.2
Hydrolyzed casein	100	13.3				19.6	27.6	31.1
Difco liver	100	8.1				35.5	58.7	64.0
Dialysate of bakers yeast	17.2	8.1				53.6	64.2	75.5
Dialysate of brewers yeast	19.7	9.0				46.2	59.5	72.1
Aqueous extract of malt sprouts	27.8	8.1				41.7	68.5	70.2
Ethanol extract of yellow corn	4.9	9.0		31.0	65.7	78.0	81.3	83.1
Acetone extract of yellow corn	0.8	6.6			21.2	51.2	84.0	95.8
Butanol extract of yellow corn	2.7	6.6		8.1	55.4	62.8	79.8	
Aqueous extract of wheat bran	4.1	15.5	19.7	27.8		63.8		
Ethanol extract of wheat bran	11.8	16.8		28.3	69.0	74.0		
Vitab II	100	10.9	12.4	33.2		46.5		
Ash of Vitab	6.4	3.7	2.3	3.3		4.2	1.4	0.2

* The basal medium contained 0.05 per cent hydrolyzed casein.

TABLE 2

Effects of various supplements on rate of fermentation of glucose by C. acetobutylicum

SUPPLEMENT	ORIGINAL MATERIAL IN SUPPLEMENT	SUPPLEMENT ADDED (MICROGRAMS PER ML.) AND GLUCOSE FERMENTED (PER CENT)					
		0*	1	10	100	250	500
	per cent						
Bacto peptone	100	19.6			20.0	22.4	23.8
Hydrolyzed casein	100	19.6			20.0	20.2	22.0
Difco liver	100	16.3			43.8	70.9	78.4
Dialysate of bakers yeast	17.2	20.5			58.9	91.4	95.9
Dialysate of brewers yeast	19.7	21.0			43.7	79.6	95.4
Aqueous extract of malt sprouts	27.8	16.3			37.0	86.1	83.4
Ethanol extract of yellow corn	4.9	21.0		28.6	59.8	65.3	74.1
Vitab II	100	19.9	22.3	29.5	51.6	60.8	

* The basal medium contained 0.25 per cent hydrolyzed casein.

than were alcoholic extracts of corn, but, because of the greater ease of extraction and higher yield, the former was preferred as a source material. The most satisfactory source found was

Vitab II,[3] a commercial product made by extraction of cereal grains. Although it was less active per unit weight than alcoholic extract of wheat bran, it has the advantage of being obtainable in large quantities in fairly concentrated form and of being completely water soluble. Alcoholic extracts of corn or bran gave turbid suspensions when taken up in water and consequently were difficult to work with.

TABLE 3

Activity of Vitab preparations for C. butylicum as indicated by turbidity of the cultures

SUPPLEMENT	ORIGINAL VITAB IN SUPPLEMENT	AMOUNT OF SUPPLEMENT ADDED (MICROGRAMS PER ML.) AND PHOTOMETER READINGS*			
		0†	0.1	1	10
	per cent				
Vitab II	100	91.5	91.3	93.0	47.8
Neutral ether extract	1.9	91.5	88.0	70.0	33.8
Acidic ether extract	4.4	91.5	82.0	34.1	13.8
Residue from ether extraction	93.7	91.5	88.3	88.4	81.5
Norit filtrate	1.75	91.5	88.7	87.7	90.3
Norit eluate	2.25	91.5	80.0	31.0	13.5
Ethyl alcohol soluble copper salts		90.8	37.3	20.0	15.0

* An inverse relationship exists between photometer readings and turbidity.
† Basal medium contained no casein.

Purification of the accessory growth factor for C. butylicum

The most satisfactory method for preliminary concentration of the factor was to extract a neutral aqueous solution of Vitab with ether for 70 hours to remove inactive material, and to follow this by an extraction with ether from acid solution for 100 hours to remove the growth-stimulating substance.

The active material of the acidic ether extract was completely adsorbed on norit but could be only partially regained by elution with alcohol and pyridine. While this procedure effected a certain amount of purification, a great loss in total activity occurred.

[3] The authors wish to thank Vitab Products, Inc., for the sample of Vitab used in this study.

The potency of the preparation could be increased by preparing the ethyl-alcohol soluble portion of the copper salts of the concentrate. This preparation gave maximum stimulation in a concentration of about 1 microgram per ml. and was readily detectable in a concentration of 0.01 microgram per ml. Since

TABLE 4

Effect of growth-stimulating substances for other organisms upon the growth of C. butylicum

ACCESSORY SUBSTANCES	INCREASE IN GLUCOSE FERMENTATION OVER THAT OF BASE
	per cent
Base plus indole acetic acid, 0.01 microgram per ml	−2.0
Base plus indole acetic acid, 0.1 microgram per ml	1.9
Base plus indole acetic acid, 0.5 microgram per ml	1.7
Base plus inositol, 50 micrograms per ml; nicotinic acid amide, 5 micrograms per ml; riboflavine, 0.25 microgram per ml; vitamin B_1, 0.5 microgram per ml; pimelic acid, 1 microgram per ml; amino acid mixture, 0.25 milligram per ml	2.9
Base plus sporogenes growth factor, 0.1 microgram per ml	−2.9
Base plus sporogenes growth factor, 1 microgram per ml	−1.9
Base plus sporogenes growth factor, 10 micrograms per ml	−0.2
Base plus vitamin B_6, 1 microgram per ml	−9.9
Base plus pantothenic acid, 0.1 microgram per ml	−0.7
Base plus pantothenic acid, 1 microgram per ml	1.6
Base plus β-alanine, 0.1 microgram per ml	−4.2
Base plus β-alanine, 1 microgram per ml	−2.0
Base plus β-alanine, 10 micrograms per ml	−5.6

only a small portion of the active material was recovered, this separation is of doubtful value.

The assays of the purified preparations are given in table 3.

At the beginning of the study, when crude materials were used as supplements, hydrolyzed casein was included in the basal medium in order to preclude the possibility of an amino-acid

deficiency. Since such small amounts of the best concentrate are required, it is improbable that any amino acid can be responsible for the stimulation. Furthermore, it does not seem possible that any amino acid could be present in the active ether extract.

TABLE 5

Growth stimulation of C. butylicum by active ether extracts after acetylation, alkali treatment, and bromination

TREATMENT	AMOUNT OF SUPPLEMENT ADDED	PHOTOMETER READINGS		
		Before treatment	After treatment	After acetylation and hydrolysis with NaOH
	micrograms per ml.			
Acetylation*	0	64.3	52.0	52.0
	0.1	48.2	47.1	44.8
	1	17.8	31.7	28.6
	10	13.8	13.8	14.3
Steamed one-half hour in normal NaOH	0	91.5	91.5	
	0.1	88.0	89.5	
	1	84.0	78.0	
	10	33.0	35.0	
Bromination	0	85.0	85.0	
	0.1	80.0	81.0	
	1	34.8	36.0	
	10	16.1	19.7	

* In the acetylation experiments, the basal medium contained 0.05 per cent hydrolyzed casein; in the other experiments no casein was used.

Replacement of the factor for C. butylicum

A number of known growth-promoting substances were assayed in an attempt to identify the butyl factor with one of these. The data in table 4 show that indole acetic acid, sporogenes growth factor,[4] inositol, nicotinic acid amide, riboflavin, vitamin B_1, vitamin B_6,[4] pimelic acid, β-alanine, pantothenic acid,[4] and a mix-

[4] The authors wish to thank A. M. Pappenheimer, Jr., for the sample of sporogenes growth factor used, S. Lepkovsky for the Vitamin B_6, and R. J. Williams for the pantothenic acid preparation.

ture of pure amino acids failed to replace the butyl factor. The amino acid mixture contained all the naturally-occurring amino acids except hydroxy-glutamic acid. However, this amino acid could not be the active substance as it was contained in the casein hydrolysate used in the basal medium.

Stability of the factor

The growth factor for *C. butylicum* was active after heating at 120°C. for 30 minutes and after steaming for one-half hour in normal alkali. Table 5 gives evidence of its alkali stability.

Acetylation of the growth factor concentrate yielded a product which was still active for *C. butylicum*, and the activity was practically the same after hydrolysis with NaOH. This indicated, either that the active compound did not contain a hydroxyl group and could not be acetylated, or that the acetylated product was readily hydrolyzed by the organism.

Bromination of the growth factor preparation in ether solution did not perceptibly diminish its activity.

SUMMARY

Accessory growth-promoting substances for *Clostridium butylicum* and *Clostridium acetobutylicum* have been found in liver, yeast, malt sprouts, yellow corn, wheat bran, and a commercial vitamin preparation (Vitab II).

A considerable degree of purification of the stimulatory substance for *Clostridium butylicum* was accomplished by extraction with ether from acidic solution, after a preliminary extraction with ether from neutral solution to remove inactive substances. The active material was completely adsorbed on norit but could not be completely eluted with alcohol and pyridine. Alcohol-soluble copper salts gave detectable stimulation at a concentration of 0.01 microgram per ml. and maximum effect at 1 microgram per ml.

The butyl factor can not be replaced by riboflavin, indole acetic acid, sporogenes growth factor, nicotinic acid amide, vitamin B_1, vitamin B_6, pimelic acid, inositol, β-alanine, pantothenic acid, or by a mixture of all the naturally occurring amino acids except hydroxy-glutamic acid.

The factor is stable to autoclaving, to steaming in normal alkali and to bromination.

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EFFECTS OF SULFANILAMIDE ON BRUCELLA MELITENSIS, VAR. MELITENSIS, ABORTUS, AND SUIS

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Received for publication August 10, 1938

Sulfanilamide is a valuable therapeutic agent in certain bacterial infections but is not a universal panacea, since it is of little or no benefit in some diseases. Favorable reports on the action of sulfanilamide in Brucellosis have been published in the European literature by Thevenet, Grous, Berger, Suchier, Richardson, and Francis. More recent articles by Toone and Stern have appeared in the American literature, citing cases of undulant fever apparently cured by this drug.

This work was undertaken in an attempt to determine the effect of sulfanilamide on *Brucella* organisms *in vitro* and on experimental infections in a susceptible animal. The problem was divided into four parts: (1) the *in vitro* action of sulfanilamide on *Brucella* organisms; (2) the *in vitro* action of related compounds;[2] (3) the *in vitro* action of sulfanilamide and antibrucella serum, and (4) the action of sulfanilamide on experimental Brucellosis in the guinea pig.

1. In vitro action of sulfanilamide on Brucella organisms. A one-tenth-per-cent solution of sulfanilamide (p-Aminobenzenesulfonamide) was made in beef-infusion broth, pH 7.3, sterilized by passing through a Seitz filter, and the concentration of 100 mgm. per cent checked by chemical determination.[3] Dilutions

[1] James A. Greene Research Fellow for the Study of Brucellosis.

[2] We are indebted to Mr. R. O. Roblin, Jr., of the American Cyanamid Co. for furnishing us with these related compounds.

[3] All chemical determinations were done by Dr. Ann Yates, of the Department of Biochemistry, Duke University.

of the filtrate were made up to 10 cc. with the same stock broth so that the final concentrations of the drug were 5, 10, 20, 30, 50, and 100 mgm. per cent respectively. Forty culture tubes

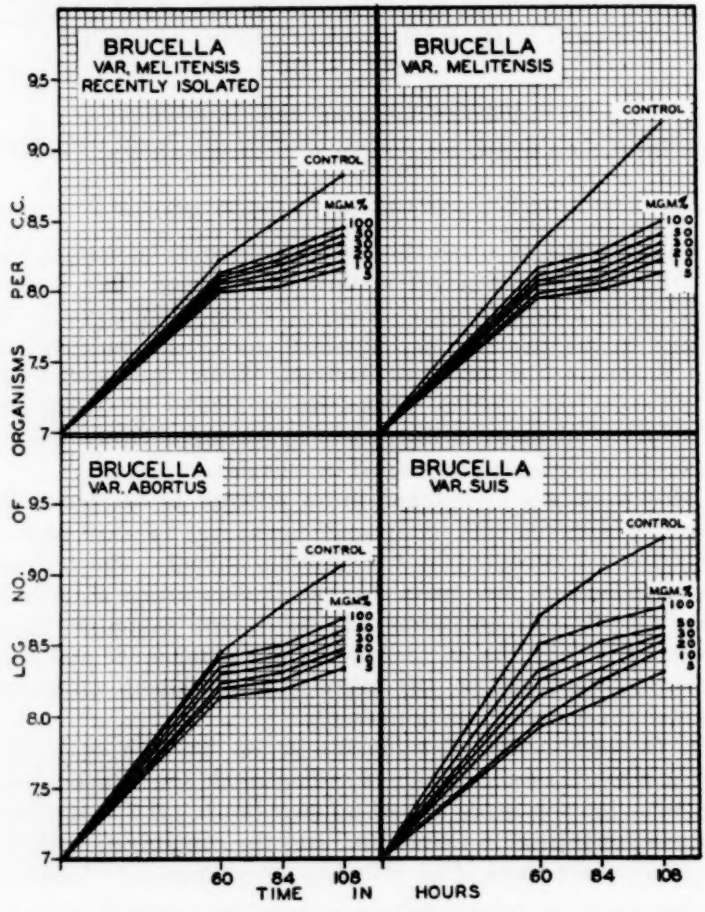

FIG. 1. GROWTH CURVES OF 3 VARIETIES OF BRUCELLA (4 STRAINS) IN DIFFERENT CONCENTRATIONS OF SULFANILAMIDE

of each concentration were inoculated by adding a suspension of 100,000,000 *Brucella* organisms (0.1 cc. of a suspension containing 1,000,000,000 organisms per cubic centimeter). Read-

ings on the Photronreflectometer (Libby) were taken after 60, 84, and 108 hours incubation at 37°C. Smears from each tube were examined after the final readings to exclude contamination, and chemical determinations showed the concentration of sulfanilamide to be unchanged and unconjugated. The pH of the media also remained unchanged (pH 7.3).

TABLE 1

Brucella organisms

HOURS	CONTROL	100 MGM. PER CENT	50 MGM. PER CENT	30 MGM. PER CENT	20 MGM. PER CENT	10 MGM. PER CENT	5 MGM. PER CENT
Variety Melitensis (428)							
60	220*	102	110	122	128	132	144
84	540	122	140	148	160	190	220
108	1180	160	190	238	270	310	390
Variety Abortus (456)							
60	258	122	136	168	175	195	210
84	520	132	164	196	212	240	320
108	1070	175	238	304	390	440	505
Variety Suis (obtained from Dr. Huddleson)							
60	490	90	130	160	172	200	320
84	1100	130	200	308	420	506	710
108	1908	210	306	412	538	644	920
Variety Melitensis (recently isolated from human)							
60	162	104	112	118	125	134	140
84	300	110	122	138	150	158	190
108	660	140	165	200	230	270	300

* Figures represent organisms in millions per cubic centimeter as determined from the average growth of forty tube cultures.

Figure 1 shows the growth curves illustrating the bacteriostatic action of various dilutions of sulfanilamide (average of 40 tubes) on 3 varieties (4 strains) of *Brucella*. Readings taken on several occasions, after 36 hours of incubation, showed no essential differences between the cultures with sulfanilamide and the controls. After 36 hours, however, the bacteriostatic effect of the sulfanilamide was very apparent (table 1). No observations on growth after 108 hours were made. Several cultures of

broth, containing 200, 300, 400, 600, and 800 mgm. per cent of sulfanilamide respectively, showed no significantly greater bacteriostatic action than the broth containing 100 mgm. per cent, and the cultures were not sterilized.

These results are similar to those obtained by Long, who showed the *in vitro* bacteriostatic effects of sulfanilamide on streptococci and other organisms. We did not confirm the work of Francis who was able to sterilize cultures of a strain of *Brucella*, var. *abortus*, by the addition of small amounts of sulfanilamide. The good results obtained by him may have been due to the small inoculum used in his experiments.

TABLE 2

Growth of Brucella, var. melitensis, after 84 hours incubation with varying amounts of sulfanilamide

DRUG	5 MGM. PER CENT	10 MGM. PER CENT	CONTROL
Sulfanilamide	200*	140	Average of 10 tubes—350
p,p′ Dihydroxydiphenyl Sulfide	0	0	
p,p′ Diaminodiphenyl Sulfoxide	300	225	
S-1	200	150	
L-167	210	145	
Disulfanilamide	190	135	

* Figures refer to millions of organisms per cubic centimeter.

2. In vitro action of related compounds on Brucella, var. melitensis. Because of the relative insolubility of the related compounds, concentrations of 5 and 10 mgm. per cent only were used. The technique was the same as described under experiment 1, and the results (table 2) indicate that the compounds tested except one (p,p′ Diaminodiphenyl Sulfoxide) had no greater *in vitro* bacteriostatic action than sulfanilamide.

3. Effect of specific serum on bacteriostatic effect of sulfanilamide. Osgood showed that sulfanilamide combined with specific immune sera has a greater anti-bacterial action than either alone. Rabbit serum with a high agglutinating titre (1–50,000), a strong opsonocytophagic reaction (Evans' technique), but weak bactericidal properties was diluted 1:10, 1:100,

and 1:1000 in beef-infusion broth pH 7.3, containing 10 mgm. per cent sulfanilamide. After sterilization by filtration, 40 tubes containing 10 cc. of each dilution, and controls of beef-infusion broth and beef-infusion broth plus 10 mgm. per cent sulfanilamide without serum, were inoculated with *Brucella*, var. *melitensis*. Bacterial counts were made after 60, 84, and 108 hours of incubation at 37°C., and the results expressed in table 3 show that the combination of this serum with sulfanilamide was less efficient than sulfanilamide alone.

4. Effect of sulfanilamide on experimental Brucellosis in the guinea pig. Forty healthy guinea pigs were injected intraperitoneally with 1,000,000,000 organisms of a culture of *Brucella*, var. *melitensis*, recently isolated from the blood of an acutely

TABLE 3

Brucella, var. melitensis 10 mgm. per cent sulfanilamide plus serum

HOURS	CONTROL	NO SERUM	SERUM 1:10	SERUM 1:100	SERUM 1:1000
60	200*	140	180	160	150
84	320	160	300	220	200
108	710	300	700	540	410

* Figures refer to millions of organisms per cubic centimeter.

ill patient. Five days after injection, one-half of the animals were fed sulfanilamide for a 10 day period, receiving 60 mgm. per kilo per day. The dose was then halved and continued for 10 more days. At intervals (table 4) the animals were skin tested and their blood examined for sulfanilamide concentration, agglutinin titre, and opsonocytophagic reaction. Thirty-five days after inoculation all animals were killed, carefully necropsied, and the spleens cultured on liver infusion agar. All the guinea pigs developed agglutinins and opsonocytophagic powers (tables 4 and 5), but at the end of the experimental period the average agglutinin titre of the treated group had fallen and the opsonocytophagic reaction had increased. The agglutinin titre of the untreated group remained high till the end of the experiment, and the opsonocytophagic reactions were only moderately strong. *Brucella* was isolated from 19 of the 20 untreated guinea

TABLE 4

Sulfanilamide treated guinea pigs

Agglutinin titre, opsonocytophagic, skin test, and blood concentration of sulfanilamide in 20 guinea pigs infected with *Brucella*

GP	15 DAYS AFTER INJECTION				25 DAYS AFTER INJECTION				35 DAYS AFTER INJECTION				SPLEEN CULTURE
	Agglutinins	Opsonocytophagic	Skin test	Blood sulf.	Agglutinins	Opsonocytophagic	Skin test	Blood sulf.	Agglutinins	Opsonocytophagic	Skin test	Blood sulf.	
				mgm. per cent				*mgm. per cent*					
1	1:160	Weak	0	18	1:160	Moderate	0	10	1:20	Strong	0	0	0
2	1:320	Weak	0	13	1:320	Very strong	0	8	1:40	Very strong	+	0	0
3	1:80	Moderate	0	14	1:160	Strong	0	7	1:20	Strong	+	0	0
4	1:80	Weak	0	19	1:80	Moderate	0	11	1:40	Strong	0	0	0
5	1:160	Weak	0	23	1:80	Strong	0	17	1:20	Very strong	0	0	0
6	1:80	Weak	0	12	1:80	Strong	0	4	1:20	Strong	+	0	0
7	1:160	Weak	0	18	1:160	Moderate	0	6	1:20	Strong	0	0	0
8	1:320	Moderate	0	16	1:320	Strong	0	6	1:20	Strong	0	0	0
9	1:160	Moderate	0	14	1:320	Strong	0	5	1:40	Very strong	0	0	0
10	1:320	Weak	0	13	1:160	Moderate	0	7	1:20	Very strong	0	0	0
11	1:20	Weak	0	11	1:20	Weak	0	7	1:20	Weak	+	0	+
12	1:320	Moderate	0	19	1:320	Strong	0	9	1:80	Strong	0	0	0
13	1:80	Weak	0	17	1:160	Moderate	0	8	1:20	Strong	+	0	0
14	1:160	Moderate	0	16	1:320	Moderate	0	11	0	Strong	0	0	0
15	1:160	Moderate	0	12	1:80	Very strong	0	10	0	Very strong	0	0	0
16	1:80	Weak	0	18	1:40	Moderate	0	6	1:20	Very strong	+	0	0
17	1:320	Moderate	0	20	1:640	Strong	0	8	1:80	Strong	+	0	0
18	1:160	Weak	0	14	1:320	Very strong	?	6	0	Very strong	0	0	0
19	1:160	Weak	0	9	1:80	Moderate	0	3	1:40	Very strong	0	0	0
20	1:320	Moderate	0	13	1:320	Strong	0	4	1:640	Weak	+	0	0

pigs and from only 1 of the 20 treated animals. The guinea pig in the latter group which had a positive culture never developed any marked serologic reaction.

TABLE 5

Agglutinin titre, opsonocytophagic, and skin test in 20 guinea pigs infected with Brucella

GP	15 DAYS AFTER INJECTION			25 DAYS AFTER INJECTION			35 DAYS AFTER INJECTION			SPLEEN CULTURE
	Agglutinins	Opsonocytophagic	Skin test	Agglutinins	Opsonocytophagic	Skin test	Agglutinins	Opsonocytophagic	Skin test	
21	1:320	Weak	0	1:1280	Moderate	+	1:640	Strong	++	0
22	1:180	Weak	0	1:320	Weak	0	1:640	Weak	0	+
23	1:80	Weak	0	1:640	Weak	+	1:1280	Moderate	+++	+
24	1:80	Weak	0	1:160	Weak	+	1:320	Moderate	++	+
25	1:80	Weak	0	1:160	Weak	+	1:640	Weak	++	+
26	1:80	Weak	0	1:320	Weak	0	1:320	Weak	0	+
27	1:320	Weak	0	1:320	Weak	+	1:640	Moderate	+++	+
28	1:160	Weak	0	1:160	Weak	0	1:320	Weak	0	+
29	1:40	Weak	0	1:80	Moderate	0	1:640	Moderate	+	+
30	1:160	Weak	0	1:320	Weak	0	1:640	Moderate	+	+
31	1:320	Weak	0	1:160	Weak	0	1:160	Weak	0	+
32	1:40	Weak	0	1:40	Weak	+	1:320	Weak	++	+
33	1:20	Weak	0	1:80	Weak	0	1:160	Weak	0	+
34	1:320	Negative	0	1:320	Weak	0	1:320	Weak	0	+
35	1:160	Weak	0	1:80	Weak	0	1:640	Moderate	++	+
36	1:320	Weak	0	1:640	Moderate	+	1:1280	Weak	+	+
37	1:40	Negative	0	1:320	Weak	+	1:640	Moderate	+++	+
38	1:80	Weak	0	1:640	Weak	0	1:1280	Moderate	0	+
39	1:160	Weak	0	1:320	Weak	0	1:640	Weak	0	+
40	1:160	Weak	0	1:320	Weak	0	1:320	Weak	+	+

DISCUSSION

In reviewing the literature, one is impressed by the fact that in the cases of Brucellosis reported to date, every patient who responded well to sulfanilamide therapy had a high agglutinin titre before the chemo-therapy was begun. Bethoux reported a patient with *Brucella* bacteremia who had a weak agglutinin titre, and he was unable to sterilize the circulating blood by sulfanilamide therapy. Our findings in guinea pigs support the clinical observations in suggesting that the bacteriostatic action

allows the normal defense mechanisms of the body to cope adequately with the invading bacteria.

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CULTURAL REQUIREMENTS FOR THE PRODUCTION OF BLACK PIGMENTS BY BACILLI

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Received for publication August 12, 1938

Spore-bearing aerobic bacteria[1] capable of producing striking black pigments have been known since the early reports of Gorini (1896) on "*Bacillus lactis-niger*" and of Biel (1896) and Lunt (1896) on "*Bacillus mesentericus-niger.*" Although these types have become established in the bacteriological literature under the respective binomial designations of *Bacillus niger* (Migula, 1900) and *Bacillus aterrimus* (Lehmann and Neumann, 1896), there has been little agreement upon their characterization or upon the cultural conditions necessary for their pigmentation.

Ford (1927) noted that *B. aterrimus* blackened potato very strikingly, in contrast to the brown color produced by *B. niger*. On the other hand, the differential key in Bergey's Manual (1934) employed the blackening of potato in the reverse order for separating the two species—*B. aterrimus*, white to pink; *B. niger*, black. Levine and Soppeland (1926) found both species capable of blackening potato, but only *B. niger* capable of fermenting lactose. This character was therefore used to separate the two species. Lehmann, Neumann and Breed (1931) considered the pigment-producing abilities of these organisms to be variable or inconstant, and grouped them together with forms closely related to *Bacillus vulgatus*.

[1] The authors wish to express their appreciation to all those who so kindly furnished the cultures which made this study possible and to Charles Thom, principal mycologist in charge, for his interest and coöperation during the course of this work.

Other species capable of producing a black coloration have been described. Carbone and associates (1921) refer to *Bacillus tyrosinogenes* of Rusconi; Fabian and Nienhuis (1934) found *Bacillus nigrificans* in spoiled pickles; and Cameron, Esty and Williams (1936) described *Bacillus betanigrificans* capable of blackening beets and culture media in the presence of iron. The relationships of these proposed species to *B. niger* and *B. aterrimus* have not hitherto been defined.

CULTURES STUDIED

Observations reported in this paper were made upon a collection of 40 cultures, 12 of which were received as named species, 7 were received unnamed but known to produce pigment, and 21 were isolated by the authors. These cultures are listed below under the three species into which they naturally fall because of their physiological characteristics. The name under which the culture was received and the history as far as known, is given opposite the number of the culture as it exists in our collection.

I. *Bacillus niger*

220 *B. mesentericus*, var. *niger*, from AMNH (733) in 1923; Kral.
229 Black bacillus, Smith, isolated in 1912.
254 *B. lactis-niger*, from Gorini (2) in 1936.
264 *B. niger*, from Hall (799) in 1936; Ford.
265 Black bacillus, from Hall (1509) in 1936.
650 *B. niger*, from Cameron in 1937; Breed; NCTC 2736; Gorini.
651 *B. niger*, from Cameron in 1937; Breed; NCTC 2592; Ford (6).
655 *B. lactis-niger*, from Cameron in 1937; Breed; Gorini (2).
18 isolates from soil and air, 1936.

II. *Bacillus aterrimus*

230 Black bacillus, from Thom in 1936 (isolated in 1912).
259 Black bacillus, from Hall (581) in 1936.
260 Black bacillus, from Hall (620) in 1936.
261 Black bacillus, from Hall (621A) in 1936.
262 Black bacillus, from Hall (622) in 1936.
353 *B. tyrosinogenes* (Rusconi), from Hall in 1937; Istituto Sieroter., Milan.
624 *B. nigrificans*, from Porter in 1937; Fabian.

653 *B. aterrimus*, from Cameron in 1937; Breed; NCTC 2591; Ford (5B)
659 *B. nigrificans*, from Cameron in 1937; Fabian.
3 isolates from soil, 1936.

III. *Bacillus betanigrificans*

648 *B. betanigrificans*, from Porter in 1937.
649 *B. betanigrificans*, from Cameron in 1937.

In addition to these cultures, 6 cultures were received as *B. aterrimus* and 6 as *B. niger* (or *B. lactis-niger*), which could not be identified as belonging to either of these species.

INFLUENCE OF THE MEDIUM ON PIGMENTATION BY B. NIGER

The importance of the substrate in the development of pigment by *B. niger* was evident when cultures, previously coal-black, failed to produce pigment when transferred to another lot of nutrient agar. These cultures, however, readily produced pigment when returned to the original medium. To overcome the supposed deficiency in the one medium, various modifications, such as the addition of carbohydrates or mineral salts, singly and in combination, or the changing of the pH value, were made without success. Finally, all brands of commercial peptones available in this laboratory were used in agar. On 5 peptone agars, *B. niger* produced blackening (see table 1) but failed to do so on the remaining 4; *B. aterrimus* failed on all. The addition of beef-extract paste to 2 of the 4 peptone agars not blackened by *B. niger* resulted in blackening; the growth of *B. niger* upon beef-extract agar alone, however, was colorless.

The addition of glucose or maltose inhibited the production of pigment on those peptone agars which were normally blackened by *B. niger*. This suggested that certain break-down products of protein were necessary for the blackening and that fermentable carbohydrates spared the protein from decomposition. Since the various commercial peptones are known to vary in their amino-acid content as well as in other respects, correction of those peptones unsuitable for pigmentation was attempted. Glycine, alanine, aspartic acid, leucine and tyrosine were added singly to

nutrient agar prepared from peptone "A"[2] *B. niger* produced a black pigmentation only on the nutrient agar to which tyrosine had been added. The addition of glucose or maltose to this medium did not interfere with the development of the pigmentation as it did in the cases mentioned above.

In addition to those media already mentioned, milk agar (equal volumes of skim milk and 4 per cent agar-agar) is suitable for *B. niger*, the black pigment usually appearing in a few days.

TABLE 1

Pigmentation responses of B. niger and B. aterrimus on various peptone agars

PEPTONE	1.0% PEPTONE		0.5% PEPTONE 0.3% BEEF EXTRACT		0.5% PEPTONE 0.3% BEEF EXTRACT 1.0% GLUCOSE	
	B. niger	*B. aterrimus*	*B. niger*	*B. aterrimus*	*B. niger*	*B. aterrimus*
A	−*†	−‡	−†	−‡	−†	+
B	+	−	+	−	−	+
C	+	−	+	−	−	+
D	+	−	+	−	−	+
E	+	−	+	−	−	+
F	−	−	+	−	−	+
G	−	−	−	−	−	+
H	+	−	+	−	−	+
I	−	−	+	−	−	+

* + black pigment present; − black pigment absent.
† Blackening with free tyrosine added.
‡ No blackening with free tyrosine added.

RÔLE OF FERMENTABLE CARBOHYDRATES IN THE PIGMENTATION OF B. ATERRIMUS

The reports of many previous workers that carbohydrate compounds are especially desirable for the black pigmentation of *B. aterrimus* have been confirmed. *B. aterrimus* cultures failed to show blackening on any of the peptone, peptone-beef, or tyrosine-peptone-beef agars blackened by *B. niger*. However, the addi-

[2] None of our cultures of *B. niger* blackened nutrient agar containing this peptone (Difco's Bacto-Peptone). Of the 9 peptones studied, nutrient agars prepared with Difco Bacto-Tryptone or Bacto-Proteose Peptone were the most readily blackened by *B. niger*.

tion of a fermentable carbohydrate to any type of peptone agar that would support at least a moderate growth of *B. aterrimus* rendered that medium favorable for pigmentation by this species, regardless of whether or not the peptone was a type blackened by *B. niger*. The differences in the types of media which permit pigmentation of *B. aterrimus* and *B. niger* are shown in table 1.

Broquin-Lacombe (1913) recognized that *B. aterrimus* had the ability to blacken an inorganic nitrogen medium containing a fermentable sugar, whereas *B. niger* lacks this ability. We have noted blue or black pigments in *B. aterrimus* cultures upon monobasic ammonium phosphate agar with such carbon sources as xylose, arabinose, glucose, levulose, maltose, sucrose, starch, dextrin, salicin, or mannitol.

All cultures of *B. aterrimus* and the majority of cultures of *B. niger* blackened sterilized potato. In view of the differences obtained on other media, it is probable that *B. aterrimus* produces pigment on potato by means of a different enzyme system than does *B. niger*. Muschel (1922) expressed the opinion that *B. aterrimus* produces a polyphenyloxydase blackening of carbohydrate condensation products. It is probable that *B. niger* produces a melanin-type pigment by means of a tyrosinase system. For either system, potato, or peptone agar containing both fermentable sugar and free tyrosine, serve as satisfactory substrata for pigment production.

Although we are in agreement with Levine and Soppeland (1926) that either species may produce blackening on potato, we do not agree that lactose fermentation by *B. niger* suffices to separate it from *B. aterrimus*. For this determination the synthetic carbohydrate agars with brom-cresol-purple indicator were used as recommended in the Manual of Methods for Pure Culture Study, edited by the Committee on Bacteriological Technique of the Society of American Bacteriologists. Growth and more or less acid formation within 2 weeks indicated a positive reaction, whereas no growth or a very scant growth and no acid indicated a negative reaction. All of our isolations of *B. niger* are lactose negative, as was the *B. niger* (culture 254) received directly from Gorini in 1936. On the other hand, cultures of *B. niger* from the

American Type Culture Collection (culture 220) and from Hall (culture 264) were lactose positive. All cultures of *B. aterrimus* were lactose negative. Therefore, lactose fermentation does not appear an adequate differential criterion. It has been previously reported (Clark and Smith, 1938) that several species of aerobic spore-formers regularly produce slow or mutant fermentations on certain sugars. For instance, *Bacillus megatherium* was noted to vary on mannose, *Bacillus cereus* and *Bacillus mycoides*, on sucrose, and *Bacillus vulgatus*, on lactose.

The cultures of *B. nigrificans* and of *B. tyrosinogenes* (Rusconi) were identical in every respect to type cultures of *B. aterrimus*; they produce similar blackening on sugar media, and are considered to be synonymous.

CULTURES DISTINCT FROM B. NIGER AND B. ATERRIMUS

B. betanigrificans exhibited morphologic and cultural differences, especially the production of gas on carbohydrate media which readily separates it both from *B. niger* and *B. aterrimus*. It was not found to produce a black pigment on potato, milk agar, or ordinary peptone or sugar agars, but it does produce a brownish or black pigment in the presence of metallic iron in various media, as originally reported by Cameron, Esty and Williams (1936).

As stated in the forepart of this paper, a total of 12 cultures received as *B. aterrimus* or *B. niger* were identified as species other than either of these. The donor of certain cultures stated that he had never seen any pigmentation with those particular cultures, but that they undoubtedly did produce color when they were isolated. This apparent variability in pigment production has led some bacteriologists[3] to believe that this character is transient, whereas others believe it a stable character, although at the moment lacking proof for their opinion except as they have observed their own cultures. Of these 12 mislabelled cultures, only 3 were identified as *Bacillus vulgatus*, a closely allied species, whereas 9 were distinctly different in their reactions.

Cultural similarities between *B. vulgatus*, *B. niger* and *B.*

[3] Personal communications.

aterrimus made it desirable to determine whether pigment production was a stable character and whether variants might be developed. Consequently, cultures of *B. vulgatus* were subcultured repeatedly in enrichment broths without any pigmented strains being obtained. Attempts were also made to produce non-pigmented strains from cultures of *B. niger* and *B. aterrimus*. Cultures were aged in acid, neutral or alkaline broths (pH 5.5, 7.0, 8.0) and plated out at varying intervals. Smooth and rough colony forms were picked from these platings for further aging and plating. In no case was it found possible to obtain a non-pigmented daughter strain from an originally pigmented parent culture. Finally, it may be stated that *B. niger* (number 229) and *B. aterrimus* (number 230) have been maintained in this laboratory for 26 years without any special care and without loss of their characteristic type of pigmentation.

SUMMARY

Bacillus niger produces a black pigment upon protein media which contain free or metabolically available tyrosine. Some commercial peptones containing no readily available tyrosine are not blackened unless free tyrosine is added. The addition of a fermentable sugar to many protein media normally blackened by *Bacillus niger* inhibits pigmentation unless free tyrosine is added.

Bacillus aterrimus blackens media containing fermentable carbohydrates, either in the presence or absence of tyrosine, but does not blacken sugar-free peptone media which are readily blackened by *Bacillus niger*. Carbohydrate media containing mineral nitrogen are also blackened by *Bacillus aterrimus* but not by *Bacillus niger*.

Cultural requirements for the production of pigment by *Bacillus aterrimus* and *Bacillus niger* are believed sufficient to warrant their recognition as separate species. Since non-pigmented variants could not be developed and since a culture of each has been maintained for 26 years without loss of pigmentation, it seems that this is a stable character.

Bacillus nigrificans (Fabian) and *Bacillus tyrosinogenes* (Rus-

coni) were found similar in all respects to *Bacillus aterrimus*, and are believed to be synonymous with that species.

Bacillus betanigrificans produces black coloration in the presence of metallic iron, but not upon the media readily blackened by the species considered above. It possesses morphological and cultural properties that easily separate it from *Bacillus niger* and *Bacillus aterrimus*.

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FACTORS LIMITING BACTERIAL GROWTH

IV. The Age of the Parent Culture and the Rate of Growth of Transplants of Escherichia Coli

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Received for publication August 15, 1938

Contrary to the earlier idea that the initial lag in the multiplication of bacteria represents a period of latency, it is now recognized that actually this is a period of intense growth during which the cells are increasing in size and metabolic activity (Huntington and Winslow, 1937). Nevertheless, the new facts have been regarded as manifestations of rejuvenescence, and the description of lag as an interval of preparation for active multiplication has been retained (Topley and Wilson, 1936). Obviously, if it is to conform to the usual definitions, rejuvenescence of bacterial cells must be marked by an increased capacity for growth and metabolism which is independent of the increase in size. We have already shown to our satisfaction that when environmental conditions are held constant, the age of the source culture of *Escherichia coli* does not influence the rate of oxygen consumption per unit of bacterial substance (Hershey and Bronfenbrenner, 1938). At the same time indirect evidence was obtained that the initial rate of growth of cells transplanted from young and old cultures is the same, and this was substantially confirmed by the observation that under favorable conditions growth in broth regularly occurs without lag, if changes in the size of cells are taken into account (Hershey, 1938). The experiments to be reported at the present time are concerned with the direct measurement of the rate of increase in bulk of bacterial substance in relation to the age of the culture of *E. coli*.

The few data of this kind contained in the literature do not

bear directly on the question of age. However, certain observations on the rate of growth during the phase of lag should be referred to. Henrici (1928) constructed curves representing the growth in total cell length of microcolonies of *Bacillus megatherium*, and noted a marked shortening of the lag period as compared with curves representing the uncorrected cell count. A similar peculiarity was found by Alper and Sterne (1933) in curves obtained by photo-electric measurement of the opacity of cultures of *Salmonella gallinarum*.

Adolph and Bayne-Jones (1932) and Bayne-Jones and Adolph (1933) computed rates of volume increase of individual cells from motion picture photomicrographs of agar cultures of *B. megatherium* and *E. coli*. Occasional cells initiated growth at the maximal rate, but lag was noticeable in most cases. The considerable differences in behavior of individual cells were not clearly correlated with the age of the parent culture.

Coombs and Stephenson (1926) have described a gravimetric method for determination of bacteria, but this appears not to be applicable to measurement of initial rates of growth. A similar method has been used by Bach (1937), however, in following the development of *Staphylococcus aureus*. This author obtained curves of increase in bacterial mass which he believed to be atypical, a conclusion evidently proceeding from misinterpretation of data. No observations of the initial growth period were made.

The observation of lag has thus been the rule whatever the method of measuring rate of growth, and the inference has usually been made that the early increase in rate is a reflection of intrinsic changes in cell physiology. Our previous experiments with *E. coli* (*loc. cit.*) have failed throughout to confirm these suggestions, and the results reported below seem to give decisive support to our earlier evidence that the age (i.e., phase of growth) of cells is not one of the factors influencing the rate of growth of this organism. Lag, when it is observed, we are inclined to attribute to initially unfavorable conditions of growth quite distinct from any peculiarity inherent in the cells.

In the following experiments we have applied three different

methods to the measurement of growth in cultures of *E. coli*, each of which has proved to yield authentic values for total increase of bacterial substance. Since these methods were suited to the study of cultures in liquid media, the findings could be directly correlated with existing knowledge of bacterial multiplication. And since the rates obtained were averages exclusive of individual variations, they could be conveniently related to the age of the parent culture.

Considerable care has been exercised to obtain physiologically "young" and "old" bacteria of constant properties. The strain of *E. coli* used was a stock culture subjected to repeated plating and dried *in vacuo* in sealed ampoules by the method of Brown (1926). To insure against dissociation during the course of the work, a new transplant was made each week from the same lot of desiccated culture and maintained in the interval by daily transfer in broth. A single lot of medium, consisting of a stock solution of 10 per cent Bactopeptone, 5 per cent Difco beef extract, and M/10 phosphate buffer of the desired pH, filtered and autoclaved, and diluted to 10 volumes with sterile 0.5 per cent saline as needed, was used throughout each series of measurements. Seeded in 200 ml. amounts in one-liter Erlenmeyer flasks with 0.02 ml. (2×10^7 bacteria) of a similar culture 24 hours old, cultures yielding reproducible growth curves were obtained. For the experiments with young bacteria, cultures prepared in this manner were centrifuged approximately three hours after seeding, at which time the organisms have attained maximal size and are just entering the phase of logarithmic multiplication. For old bacteria, cultures of 24 hours or more were used.[1] Accompanying each experiment, counts of viable cells have been made to verify that the rate of multiplication corresponded with that of young or old bacteria respectively, and to compute the average size of cells. In addition a few trials were made to determine whether the organisms in the three-hour cultures would show the increased susceptibility to dele-

[1] In these, and similar unreported experiments, no significant change in growth capacity or metabolic activity has been observed in the viable cells remaining in cultures from one to seven days old.

terious influences commonly ascribed to "physiologically young" cultures (Sherman and Albus, 1923). In every case these various evidences fully justified our choice of conditions for obtaining young and old bacteria. (See tables 1, 3 and 4.)

THE ASSAY OF BACTERIAL SUBSTANCE

Total bacterial substance was estimated for measurement of growth rate by means of a photo-electric nephelometer suited to quantitative measurements (Stier, Arnold, and Stannard, 1933–4), by determination of centrifugable nitrogen (Mueller, 1935), or by a manometric method.

The first of these was found to be the most convenient, and, when used with the proper precautions, extremely accurate.

TABLE 1

Survival of young and old bacteria in distilled water

AGE OF CULTURE	DILUTION	DILUTING FLUID	NUMBERS PER ML.* AFTER			
			0 minutes	15 minutes	30 minutes	60 minutes
hours						
24	10^{-7}	Distilled water	109	113	85	55
3	10^{-4}	Distilled water	90	1	0	0
3	10^{-4}	Saline	88	90	87	79

* Average of three plates.

The recommendations of the author regarding the method of expressing optical density, and of calibration, were followed in detail. The excellent discussion of this instrument by Longsworth (1936) was also found helpful. It was convenient to make the calibration using one tube for the blank containing broth, and reserving a second for the sample of culture. In every case the reading of the latter was preceded and followed by a reading of the blank, all three readings being repeated if necessary until the first and last were in agreement. Calibration was made with dilutions in broth of a formalinized culture of *E. coli* which had been incubated 24 hours and standardized by plate count. The turbidity of formalinized cultures did not change during several hours. The instrument was suitable for

measuring populations of 10^7 to 10^9 organisms of minimum size (10^6 to 10^8 in young cultures) per milliliter, giving values reproducible within less than two per cent in the region of 10^8 per milliliter. In the present experiments, cultures were diluted, if necessary, with broth to approximate this number before measurement.

Since the optical density of bacterial suspensions may vary with factors other than the size and number of organisms, preliminary experiments were necessary to ascertain the validity of interpreting as bacterial mass the turbidity of cultures of different age in terms of a single calibration. For this purpose, suspensions of organisms washed in saline were prepared from three-

TABLE 2

Comparison of optical density with nitrogen content of suspensions of young and old cells

AGE OF SOURCE CULTURE	VIABLE* COUNT (VC)	NEPHELOMETRIC* COUNT (NC)	NITROGEN* PER ML. (N)	APPARENT SIZE (NC/VC)	RATIO (N/NC)
hours	$\times 10^8$ *per ml.*	$\times 10^8$ *per ml.*	*mgm.*		
24	940	835	0.025	0.9	0.30
3.5	310	739	0.025	2.4	0.34

* Cells washed twice and resuspended in sufficient saline to give a turbidity equal to 10^9 organisms per ml.

and-one-half and twenty-four hour cultures respectively, and the bacterial content of each was determined by parallel measurements of bacterial nitrogen and optical density. In addition the suspensions were counted to provide a measure of relative size.[2] Table 2 sets forth the results of this experiment, in which it can be seen that the ratio of turbidity to nitrogen is constant within expected error in spite of the three-fold difference in aver-

[2] Since measurements of size are based on viable counts, the values are valid only for suspensions of viable organisms. There is no indication that our 24-hour cultures contain appreciable numbers of non-viable cells (see, for example, an earlier experiment, Hershey, 1937). Nor is it probable that considerable numbers of cells from young susceptible cultures die during the process of washing, since in actual test cultures of this age diluted largely in saline were found to be 90 per cent viable after one hour at room temperature, in contrast to their mortality in water (table 1).

TABLE 3

Initial growth rates in broth at pH 7.4 of E. coli from cultures of different age

A. Nitrogen determinations

DATE OF EXPERIMENT	PARENT CULTURE		SUBCULTURE								
	Age	Viable count	Period of incubation	Initial* viable count	Final viable count	Initial* N/ml.	Final N/ml.	Initial N/cell	Final N/cell	Rate of multiplication	Rate of growth†
	hours	*per ml.*	*hours*	$\times 10^{8}$/*ml.*	$\times 10^{8}$/*ml.*	$\times 10^{-4}$ *mgm.*	$\times 10^{-4}$ *mgm.*	$\times 10^{-12}$ *mgm.*	$\times 10^{-12}$ *mgm.*	*gen./hr.*	*per hr.*
3/16/38	24	2.2×10^{9}	1.58	2.2	58.0	0.57	21.0	0.26	0.36	0.7	3.3
3/17/38	3	7.4×10^{8}	1.50	0.37	51.0	0.49	14.0	1.32	0.28	3.4	3.2

B. Manometric measurements

DATE OF EXPERIMENT	PARENT CULTURE		SUBCULTURE								
	Age	Visible count	Period of incubation	Initial* viable count	Final viable count	Initial* RO_2/ml.	Final RO_2/ml.	Initial RO_2/cell /hour	Final RO_2/cell /hour	Rate of multiplication	Rate of growth‡
	hours	*per ml.*	*hours*	$\times 10^{8}$/*ml.*	$\times 10^{8}$/*ml.*	$mm^3./hr.$	$mm^3./hr.$	$\times 10^{-7}$ $mm^3.$	$\times 10^{-7}$ $mm.^3$	*gen./hr.*	*per hr.*
3/20/38	24	2.0×10^{9}	1.25	20.0	39.0	4.05	35.4	2.0	9.1	0.8	2.5
4/ 5/38	36	1.5×10^{9}	1.50	1.5	5.0	0.45	9.0	3.0	16.3	1.2	2.9
3/18/38	72	1.2×10^{9}	2.33	1.2	20.0	0.21	13.5	1.8	6.8	1.7	2.6
3/18/38	3.3	2.0×10^{7}	1.17	2.0	28.0	1.35	15.6	6.8	5.6	3.3	3.0

C. Nephelometric measurements

DATE OF EXPERIMENT	PARENT CULTURE		SUBCULTURE								
	Age	Viable count	Period of incubation	Initial* viable count	Final viable count	Initial§ nephelometric count	Final§ nephelometric count	Initial size¶	Final size¶	Rate of multiplication	Rate of growth‖
	hours	*per ml.*	*hours*	$\times 10^6$/*ml.*	$\times 10^6$/*ml.*	$\times 10^6$/*ml.*	$\times 10^6$/*ml.*			*gen./hr.*	*per hr.*
2/24/38	24		3.00			0.1	79.0				3.1
3/13/38	24	1.3×10^9	2.50	0.13	4.5	0.2	41.0	1.4	9.1	2.1	3.1
3/16/38	24	2.2×10^9	1.58	2.2	4.0	2.1	58.0	1.0	6.0	0.7	3.1
4/ 5/38	36	1.5×10^9	1.50	1.5	5.5	1.7	32.0	1.2	6.0	1.2	2.8
3/18/38	72	1.2×10^9	2.33	1.2	20.0	2.0	97.0	1.6	4.8	1.7	2.4
4/ 5/38	24	1.9×10^9	0.75	9.7	9.3	7.5	34.0	0.8	3.7	0.0	2.9
3/20/38	24	2.0×10^9	1.25	20.0	39.0	16.0	18.0	0.8	4.5	0.8	2.8
3/21/38	24	5.0×10^9	0.50	50.0	41.0	47.0	80.0	0.9	2.0	0.0	2.1
3/24/38	36	1.0×10^9	0.50	26.0	26.0	28.0	64.0	1.1	2.5	0.0	2.4
3/ 3/38	3.0	1.3×10^7	2.00	0.13	17.0	0.8	80.0	6.1	4.7	3.5	3.3
3/17/38	3.0	7.4×10^6	1.50	0.37	6.8	2.9	51.0	8.0	4.5	3.4	2.8
3/18/38	3.3	2.0×10^7	1.17	2.0	28.0	9.7	110.0	4.8	3.9	3.3	3.0

* Values obtained by analysis of the parent culture, corrected for the dilution resulting when the subculture was seeded.

† Two-fold increases in bacterial nitrogen per hour.

‡ Two-fold increases in rate of O_2-use per hour.

§ Turbidity expressed in terms of calibration with 24-hour culture.

¶ Ratio of nephelometric to viable count (24-hour cells = ±1.0).

‖ Two-fold increases in turbidity per hour.

age size of cells in the two suspensions. The nephelometric method is, therefore, suitable for the estimation of bacterial mass in cultures of different age, a conclusion which might also be made from the agreement between growth rates obtained by the various methods (table 3).

The method used for determining bacterial nitrogen has already been described (Hershey, 1937). The principal source of error with this method is in centrifugation. Recentrifuging in conical tubes is essential. Populations of 10^7 per ml. or greater may be satisfactorily determined, the micro-Kjeldahl method requiring about 200 ml. of the three-hour cultures, and as little as 10 ml. (0.3 mgm. N) of cultures fully grown.

The use of the Warburg-Barcroft manometer in our hands has been referred to (Hershey and Bronfenbrenner, 1937 and 1938). Bacteria in young cultures were estimated by measuring the oxygen consumption of five ml. aliquots without added nutrient, after determining that the oxygen use under these conditions was not different from that in fresh broth. This has been found to hold substantially true for cultures in which the population has not reached 10^9 organisms per milliliter. For the estimation of bacteria in older cultures, one ml. of the uncentrifuged culture was placed in the side arm of the manometer vessel, with 4 ml. of broth on the floor. In either case, after temperature equilibration, the side-arm was tilted and the oxygen consumption at zero time obtained in the usual way. This method was applicable to measurement of 10^8 or more organisms of minimum size per milliliter. Values obtained in this way represent, of course, the bacteria present at the time of reading, rather than at the time of removal from the parent culture.

NEPHELOMETRIC MEASUREMENT OF RATE OF GROWTH

Broth in 100 ml. amounts, filtered asceptically through paper into a one-liter Erlenmeyer flask, was warmed to 37°C. in a water bath and seeded with the desired aliquot of the parent culture. The latter was prepared as previously described from a seeding of 10^5 organisms per milliliter. If the final population for the

growth rate measurement was to exceed 10^8 organisms per milliliter, aeration was practised to avoid suppression of growth due to lack of oxygen. Immediately after making the transfer, the parent culture was counted, and directly killed by addition of 0.5 per cent formalin. Turbidity of the formalinized sample was measured within 10 minutes. At the end of the incubation period the subculture was analyzed in the same way. From the values obtained for the two cultures, the rate of growth was expressed by the formula $n/t = \frac{\log b - \log B}{t \log 2}$, where n/t is the reciprocal of the apparent generation time, B is the initial and b the final number of bacteria, and t the period of incubation, these values referring to the daughter culture. Substitution into this formula of the viable counts gave the rate of multiplication in generations per hour, while the nephelometric values gave the rate of mass increment.[3] Since the turbidity of young cultures is slightly below the value expected from the nitrogen content (table 2), the tendency of error from this source is to give abnormally low values for the growth rates of old cells. The ratio of the nephelometric to the viable count was taken as the index of size. The nephelometer having been calibrated with 24-hour cultures, the index is near unity for cultures of this age and proportionately larger for the young cultures, with a maximum value approaching ten.

MEASUREMENT OF RATE OF GROWTH BY DETERMINATION OF BACTERIAL NITROGEN

This method has been used primarily as a control corroborating nephelometric experiments. The procedure described above was followed exactly, with the exception that nephelometric measure-

[3] The growth rate calculated from nephelometric data may be defined as the number of two-fold increases in turbidity per hour, and would be identical with the multiplication rate (generations per hour) in a hypothetical culture in which the average size of cells remained constant. This unit has been chosen to give values numerically comparable with the multiplication rate. Values given by the formula we have used can be readily converted to those commonly expressed by the equation $C_r = 1/t \ln b/B$ to which they are proportional, by applying the factor 0.69.

ments were supplemented by determinations of the nitrogen in the washed sediment from the formalinized cultures. Growth rates were computed in the same way as from the nephelometric data. Size was expressed as nitrogen per viable cell, which was found to be about 0.3×10^{-10} mgm. for 24 hour cultures, with a maximum of 10 times this figure for young cultures.

MANOMETRIC MEASUREMENT OF GROWTH RATE

This method provided a valuable check on the two preceding methods, since it is not subject to possible errors due to formation of non-bacterial sediments in the medium. A systematic error is introduced, however, from the fact that removal of carbon dioxide in the respirometer vessel depresses the rate of growth during the interval of equilibration of temperature. This effect tends to cancel out when young cultures are being studied, and to give somewhat low values for the growth rate of cultures seeded with old cells. The depressive effect of lack of carbon dioxide on the growth rate was found to be less marked the larger the numbers of bacteria in the vessel, and introduced no significant error under the conditions used when 10^8 or more bacteria per ml. were present.

The manometric measurement of growth rate differed in detail from the two preceding methods. Aliquots of the parent culture representing 10^8 to 10^9 bacteria each were placed in duplicate vessels of the respirometer and the rate of oxygen consumption determined after equilibration of temperature. The bacterial count was made and the seed introduced into the subculture at the moment when reading of the manometers was begun, samples being taken for this purpose from the duplicate vessel. After a suitable incubation period, as the numbers approached 10^8 bacteria per ml., five ml. aliquots of the daughter culture were placed in each of the two warmed vessels and equilibrated 20 minutes in the water bath. When the temperature was constant, the viable count was made from the duplicate vessel, and readings were begun as before. The period of incubation of the subculture was thus represented by the interval between the zero times of the respective measurements. The

growth rate was obtained by substitution of these values into the formula. Size, expressed as rate of oxygen uptake per cell, was found to be about 2.0×10^{-7} mm³ per hour for 24-hour cultures, and five to eight times greater in young cultures. Maximal size is difficult to observe by this method, for the reason that the cells are already becoming smaller when the bacteria are numerous enough to permit of measurement, but the values conform well with indices obtained at corresponding times by the other methods.

Values for the rates of multiplication and of growth determined by the three methods, together with data on population and average size in the cultures studied, are given in tables 3 and 4. Reference to the last column of table 3A, B and C, showing the rate of growth obtained by the various methods, discloses at once the substantial agreement.

In table 5 the observed values have been averaged and submitted to statistical analysis. Transplants made from the young cultures have shown only insignificantly higher rates of growth, the odds against these differences resulting from chance being about three to one and two to one, respectively, for the two experimental series. The ratio of the difference to its standard error for the first series is 1.1, for the second 0.8, while the minimum significant value is generally taken to be 3.0. On statistical reasoning alone, therefore, neglecting the tendency of known experimental errors, the smallest difference in growth rate to be regarded as significant is 0.36 generation per hour for the second series. Thus, with the average value of 21.4 minutes for the apparent generation time of old cells, that of more rapidly growing young cells should be at most 19.0 minutes. Actually, the latter value is 20.7 minutes. It will be remembered, moreover, from the discussion of the several methods, that the known errors tend in each case to give abnormally low values to the growth rate of the elder seedings, and the presence of dead cells in these seedings undoubtedly augments this tendency. The difference actually observed, about six per cent for the totaled values, is entirely within the limits of magnitude reasonably attributable to these sources of error.

TABLE 4

Initial growth rates in broth at pH 6.8 of E. coli from cultures of different ages

Nephelometric measurements

DATE OF EXPERIMENT	PARENT CULTURE		SUBCULTURE								
	Age	Viable count	Period of incubation	Initial* viable count	Final viable count	Initial§ nephelometric count	Final§ nephelometric count	Initial size ¶	Final size ¶	Rate of multiplication	Rate of growth‖
	hours	*per ml.*	*hours*	$\times 10^8$/*ml.*	$\times 10^8$/*ml.*	$\times 10^8$/*ml.*	$\times 10^8$/*ml.*			*gen./hr.*	*per hr.*
4/ 8/38	24	1.9×10^9	2.50	0.2	5.5	0.21	35.0	1.1	6.3	1.9	3.0
4/14/38	24	9.1×10^8	2.91	0.1	8.5	0.15	44.0	1.4	5.2	2.2	2.9
4/ 9/38	24	1.9×10^9	1.40	1.9	3.0	2.1	22.0	1.1	7.3	0.5	2.5
4/15/38	24	1.2×10^9	1.40	1.2	1.4	1.4	17.0	1.2	12.1	0.2	2.6
4/16/38	24	1.1×10^9	1.66	1.1	3.1	0.9	22.0	0.8	7.1	0.9	2.8
4/16/38	48	1.6×10^9	1.78	1.6	5.4	1.8	32.0	1.1	6.0	1.0	2.4
4/11/38	12	8.5×10^8	0.66	8.5	7.3	8.8	35.0	1.0	4.8	0.3	3.0
4/13/38	12	7.9×10^8	0.66	7.9	9.6	8.8	30.0	1.1	2.5	0.4	2.6
4/12/38	24	1.3×10^9	0.33	6.5	6.5	5.7	12.0	0.9	1.8	0.0	3.2
4/12/38	24	1.3×10^9	0.66	6.5	6.8	5.7	25.0	0.9	3.7	0.1	3.2
4/13/38	24	1.1×10^9	0.33	5.4	4.7	6.6	12.0	1.2	2.6	0.0	2.6
4/14/38	24	9.6×10^8	0.43	9.6	9.0	12.0	28.0	1.2	3.1	0.0	2.8
4/12/38	3.0	5.5×10^6	3.40	0.01	1.2	0.07	14.0	6.0	11.7	2.0	2.6
4/14/38	3.2	9.3×10^6	2.75	0.03	8.8	0.15	35.0	5.3	3.9	3.0	2.9
4/13/38	3.5	3.2×10^7	2.53	0.06	7.4	0.26	42.0	4.1	5.7	2.7	2.9
4/11/38	2.5	2.2×10^6	1.33	0.22	4.2	1.55	27.0	7.0	6.5	3.2	3.1
4/17/38	2.5	3.1×10^6	1.33	0.31	5.3	2.00	29.0	6.5	5.5	3.1	2.9
4/17/38	3.0	6.6×10^6	1.33	0.33	5.2	2.00	30.0	6.1	5.8	3.0	2.9
4/15/38	3.5	2.3×10^7	1.66	0.23	3.3	1.01	20.0	4.4	6.1	2.3	2.6
4/ 9/38	3.5	3.0×10^7	0.45	3.0	7.3	12.4	36.0	4.1	5.0	2.9	3.4
4/15/38	3.5	2.3×10^7	0.66	2.3	7.0	10.1	30.0	4.4	4.3	2.4	2.4

* § ¶ ‖ See table 3-c.

If it be agreed that no significant differences exist between the growth rates of young and old bacteria, the same cannot be said of the rates of multiplication. The latter, calculated for the same cultures (tables 3 and 4), illustrate very clearly the marked differences corresponding with the age of the inoculum which are commonly observed. The paradox that old and young bacteria may exhibit identical growth rates, while differing markedly in rate of multiplication, is to be explained only by consideration of average cell-size. Growing at the same rate, the smaller bacteria from old cultures require a longer time

TABLE 5

*Effect of pH and age of culture on rate of growth of transplants of E. coli**

NUMBER OF OBSERVATIONS	pH	AGE OF PARENT CULTURE	GROWTH RATE	APPARENT GENERATION TIME	DIFFERENCE	STANDARD DEVIATION	STANDARD ERROR OF MEAN	STANDARD ERROR OF DIFFERENCE	RATIO OF DIFFERENCE TO S. E.	ODDS AGAINST DIFFERENCE DUE TO CHANCE
		hours	*per hour*	*minutes*	*per cent*					
13	7.4	12–72	2.8	21.4	10.0	0.338	0.094	0.270	1.1	3:1
5	7.4	2– 3.5	3.1	19.3		0.566	0.253			
12	6.8	12–72	2.8	21.4	3.3	0.257	0.074	0.118	0.8	2:1
9	6.8	2– 3.5	2.9	20.7		0.283	0.094			

* Analysis of data of tables 3 and 4.

("lag") to reach the size at which fission occurs in freshly seeded broth, than do young bacteria of greater initial size.

Inspection of the tables reveals no systematic errors affecting the conclusions to be drawn. The two series of experiments, using broth of pH 6.8 and 7.4 respectively (table 5), have yielded identical results. The somewhat smaller experimental variation in the latter series bespeaks the accuracy which comes with practice.

It should be observed that the experiments include a rather wide range of initial seedings, and periods of incubation, neither of which has had any effect on the rate of growth. This adds considerably to the significance of the findings, for the magnitude and kind of errors expected under the several conditions

are very different. The smaller seedings were used especially to test the argument that differences in growth rate of organisms of different age might be magnified in this case, as are differences in the rate of multiplication. On the other hand, with large seedings growth could be measured over a much shorter time period, even a fraction of a single generation time, which might be expected to consist quite neatly of the period of "rejuvenescence." No sign of lag in absolute growth rate was encountered, however, in any of the experiments, the recorded values agreeing precisely with the maximum rates of growth attained in cultures observed continuously by the same methods (unpublished data). The initial growth rates measured in these experiments represent, therefore, the maximum rate for this organism under the conditions of cultivation employed, and no "period of adjustment," either of bacteria to the new medium or the reverse, is evident. The fact that the maximum absolute rate of growth is less than three "doublings" per hour, while the rate of multiplication at its maximum may exceed four generations per hour, is explained by the circumstance that the latter is observed during the period in which size is decreasing, i.e., bacteria are actually multiplying faster than they are growing.

The bearing of these observations on the general problem of growth phases is clear: that so far as differences in rate of numerical increase are concerned, "physiological youth" of the cells in young cultures of *E. coli* is an artifact, and that changes in size, conditioned in turn by changing environmental status, and the direct effect of the latter on metabolism, sufficiently explain the course of events in the development of the culture. Experiments are being planned, directed toward further elucidation of these relationships.

SUMMARY

Initial growth rates deduced from measurements of turbidity, bacterial nitrogen, and oxygen uptake of broth transplants of *Escherichia coli* seeded from young and old cultures respectively, have given identical values, while parallel measurements of rate of multiplication revealed the expected differences. We con-

clude that the physiologic state of the cells does not influence their rate of growth, and that the lag in rate of multiplication must be attributed to changes in the factors limiting the size at which fission occurs during the period of growth.

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A STUDY OF THE LIPIDS OF CERTAIN ENTERIC BACILLI[1]

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Received for publication August 19, 1938

During the past decade bacteriologists have become increasingly interested in the study of the chemical composition of bacterial cells. Numerous investigators have demonstrated a correlation between the cell components and certain biological activities. Most of the investigations have been concerned with carbohydrates, proteins, nucleic acids, *etc.*; the lipid fractions have been only slightly studied. Thus, there is a need for an intensive study of the bacterial lipids. The present paper is the first of a series of such studies.

LITERATURE

In 1893 Cramer demonstrated that the lipid content of certain common organisms varied with the carbohydrate content of the nutrient medium. Using a Friedländer's bacillus, he found that the lipid content of the organism was 10.26 per cent on nutrient agar, but on the same medium plus 5 per cent glucose, the lipid content rose to 22.68 per cent. Lyons (1897) verified this work by showing that the lipid content of an organism isolated from water, when grown on a one-per-cent glucose medium, had a lipid content of 11 per cent, but when the amount of glucose was increased to 10 per cent, the lipid content rose to 23 per cent. Nishimura (1893) also studied an organism isolated from water

[1] Based on portions of a thesis presented by Clara H. Williams, June 1938, to the University of Rochester in partial fulfillment of the requirements for the degree of Master of Science.

The study upon which this report is based was supported in part by a grant from the Rochester Branch of the American Association of University Women.

and found the total lipid to be 3.19 per cent of the dry weight of the bacteria. Approximately 0.68 per cent of the dry weight of the organism was lecithin. He isolated a fatty acid which melted at 15°C. and a mixture of fatty acids which melted at 51°C. The former, he believed to be oleic acid, the latter a mixture of palmitic and stearic acids.

Nicolle and Allilaire (1909) analyzed a series of enteric bacilli grown upon a potato agar at 37°C. for 24 hours. The total lipid content ranged from 9 to 16 per cent of the dry weight of the organisms and the presence of phosphorus in the extracts indicated that a phospholipid was probably present. Dawson (1919) found that the lipid content of *Escherichia coli* did not vary appreciably when he employed various combinations of peptone, meat extract, edestin, flour proteins, and potato juice in the nutrient media, except when one per cent glycerol was incorporated. The total lipid on the glycerol-free media varied from 4 to 5 per cent, but in the presence of glycerol it rose to 8 per cent.

Eckstein and Soule (1931) grew *Escherichia coli* on a synthetic medium containing alanine in one case and cystine in another as the source of nitrogen. In the former the lipid content of the dry cells was 7.8 per cent, in the latter the lipid content dropped to 3.6 per cent. The iodine number of the total lipid was 25.6 and that of the total fatty acids 37.4. They postulated, therefore, that oleic acid was the only unsaturated fatty acid present. They reported that a phospholipid was present to the extent of 17.4 per cent of the total lipid, but no cholesterol or other sterols were found. The absence of sterols had been observed by Miyoshi (1930). Hecht (1935), on the other hand, reported that he obtained a positive test for cholesterol with the residue, when he warmed petroleum-ether extracts of *Escherichia coli* for 8 hours.

EXPERIMENTAL

Materials and methods

Organisms. Nine strains of Gram-negative, non-sporulating, enteric bacilli were used for analysis. They represented four species or types. Of these, four strains, 214, 214PA5, 225, and

229B, were *Escherichia coli*; three strains, 234, 239-1 and 248-1, were *Escherichia communior*; one strain, 300-1, was a characteristic *Escherichia-Aerobacter* "intermediate", strain 691, (Tittsler and Sandholzer, 1935); and strain 657 was *Shigella paradysenteriae* (Flexner). Originally, strain 229B fermented sucrose, but during the past year it has lost this capacity. Organism 214PA5 is a substrain of 214. It was isolated after several serial passages of the parent strain on lithium-chloride agar (nutrient agar plus 0.3 per cent lithium chloride) in July 1936. Since then it has been maintained on plain agar.

Culture media. Throughout the present study, the organisms used for analysis were grown on plain agar having the following composition: peptone (Bacto), 20 grams; beef extract (Bacto), 3 grams; sodium chloride, 5 grams; water (distilled), 1000 cc.; final pH, 7.4 to 7.6. The agar was dispensed in Kolle flasks and Petri dishes.

The peptone was analyzed carefully for the presence of lipid. Calculations based on the analysis of 15 grams of dry peptone showed that it contained less than 0.01 per cent of total lipid. Analyses reported in the literature, (Leach and Winton, 1920), for beef extract show that it contains approximately 0.16 per cent total lipid, an amount which is negligible for the present purpose.

Stock cultures for inoculation of the agar were prepared in a medium of the following composition: peptone (Bacto), 20 grams; sodium chloride, 5 grams; water (distilled), 1000 cc.; final pH 7.4 to 7.6.

An agar medium was chosen in preference to a liquid one for growing the organisms for analysis because of the greater ease with which the organisms could be harvested in large quantities. The usual harvest of dry bacteria was approximately 2 grams from a total of 79 containers (44 Kolle flasks and 35 Petri dishes), or 2.8×10^{-3} mgm. per sq. mm.

Preparation of samples for lipid analysis. The surface of the agar medium was inoculated with a 24-hour peptone-water culture of the organism and incubated at 37°C. for 24 hours. The consequent growth was washed from the surface of the agar with sterile distilled water and the organisms were sedi-

mented by high speed centrifugation for an hour at approximately 2500 r.p.m. The sedimented residue was then frozen and dried in the frozen state *in vacuo*. The viable organisms dried in this manner were used for analysis.

Extraction of the lipids. The dry organisms were ground by hand in a mortar to a fine powder and aliquots of from 1 to 2 grams were carefully weighed in a 22 × 80 mm. extraction thimble. Extraction of the lipids was carried out using, first, alcohol (2 parts) and later ethyl ether (1 part). To shorten the time required for extraction, and to make the extraction more complete, hot alcohol was employed. The thimble was inserted in a tower above a flask containing the boiling solvent and the lipids were thus extracted continuously with fresh hot alcohol.

Determination of lipid fractions. Suitable aliquots of the alcohol-ether extract were used for determining the total fatty acids, the phospholipid, the fatty acids in the phospholipid by the oxidative methods of Bloor (1928). The iodine numbers of each of these fractions were determined according to the method of Yasuda (1931).

The percentage of phosphorus in the phospholipid was found by digesting the phospholipid with 10 N sulfuric acid and Superoxol and measuring the amount of phosphate in the resulting solution using the colorimetric method outlined by Kuttner and Cohen (1927). The percentage of nitrogen was determined by digesting the phospholipid as in the case of phosphorus and determining the ammonia formed by Nesslerization as suggested by Folin and Farmer (1912). The method was perfected, so that as little as 20 to 40 gamma could be determined by the use of a sensitive photoelectric colorimeter developed by Bloor (1938).

Qualitative tests for cholesterol and other sterols were made on the acetone soluble portion of the total lipid using the Liebermann-Burchard and the Windaus method modified by Okey (1930).

Results

The detailed data of the lipid analyses expressed as per cent dry weight of the bacteria are presented in tables 1 to 5 inclusive,

and will be discussed below under separate headings. Most of the findings are summarized graphically in figure 1. Sufficient data have been presented in each table to permit the reader to evaluate for himself the significance of the differences between organisms.

Total fatty acids. The values for total fatty acids and the respective iodine numbers are given in table 1. The data show that the total fatty acids varied from approximately 2.5 to 5.0 per cent of the dry weight of the bacteria. No correlation was

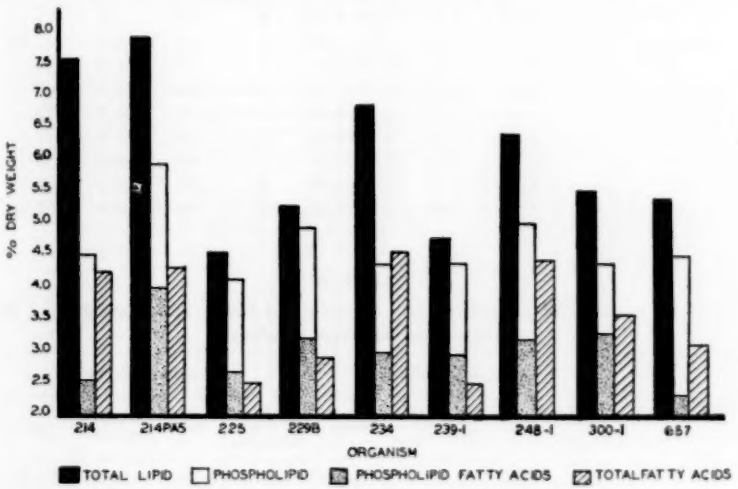

FIG. 1. SUMMARY OF ANALYSES OF BACTERIAL LIPIDS

noted between the total fatty acids and the cultural characteristics of the organisms. In general, the organisms fall into two groups, however, those having total fatty acids comprising from 4 to 5 per cent of the cell (214, 214PA5, 234, 248-1) and those whose total fatty acids represent from 2.5 to 3.0 per cent of the cell, (225, 229B, 239-1, 657). The single *Escherichia-Aerobacter* "intermediate" strain in the group has a total fatty acid content which is midway between the two groups.

The iodine numbers show a wide, uncorrelated variation, with values ranging from 42 to 82. The values are all below that of oleic acid which has an iodine number of 90. It is probable

that the only unsaturated fatty acid present is oleic, and it is probably present in varying amounts.

TABLE 1

Analysis of bacterial lipids. Total fatty acids based on dry weight and the corresponding iodine numbers

ORGANISM	PER CENT TOTAL FATTY ACIDS				IODINE NUMBERS			
	Number of determinations	Mean	Standard deviation	Range	Number of determinations	Mean	Standard deviation	Range
214	6	4.21	0.10	4.01–4.45	3	73	1.7	71–75
214PA5	4	4.27	0.10	4.14–4.38	4	48	3.7	42–52
225	3	2.51	0.14	2.47–2.56	4	49	1.0	48–50
229B	4	2.86	0.17	2.71–3.02	3	53	1.4	50–54
234	3	4.60	0.47	4.26–4.89	3	62	3.7	52–70
239-1	4	2.62	0.26	2.47–2.70	3	82	9.4	71–94
248-1	3	4.41	0.10	4.30–4.51	3	42	3.6	39–47
300-1	3	3.43	0.22	3.40–3.46	3	76	0.0	73–78
657	3	2.92	0.26	2.87–2.99	3	59	6.8	57–63

TABLE 2

Analysis of bacterial phospholipids. Phospholipid based on dry weight and the corresponding iodine numbers

ORGANISM	PER CENT PHOSPHOLIPID				IODINE NUMBERS			
	Number of determinations	Mean	Standard deviation	Range	Number of determinations	Mean	Standard deviation	Range
214	4	4.36	0.33	3.90–4.65	3	70	10.3	61–80
214PA5	4	5.66	0.33	5.51–5.85	3	52	7.2	47–57
225	3	4.07	0.10	4.02–4.14	4	47	12.0	47–50
229B	3	5.04	0.14	4.83–5.15	3	31	0.0	29–32
234	4	4.48	0.26	4.24–4.69	3	41	5.3	41–42
239-1	3	4.35	0.00	4.15–4.56	4	27	3.6	23–32
248-1	4	4.89	0.28	4.78–5.01	3	87	3.7	85–88
300-1	3	4.87	0.24	4.81–4.92	3	60	6.5	59–62
657	3	4.40	0.22	4.29–4.58	3	75	7.2	73–76

Phospholipids. The values for the phospholipid content of the dried bacteria and the corresponding iodine numbers are recorded in table 2. Apparently the phospholipid represents the major portion of the bacterial lipid. It ranges from approxi-

mately 4 to 6 per cent in this group of organisms, the total lipid varying from 4.5 to 8 per cent (table 6). The phospholipids appear to be fairly constant for the organisms.

The variation in iodine number, which ranges from 23 to 88, may have two explanations. There may be a wide variety of phospholipids present which would account for such variations, or there may be but one phospholipid, having the same degree of unsaturation, but present in varying amounts.

Phospholipid fatty acids. The data presented in table 3 indicate that the phospholipid fatty acids in these organisms are

TABLE 3

Analysis of bacterial phospholipids. Phospholipid fatty acids based on dry weight and the corresponding iodine numbers

ORGANISM	PER CENT FATTY ACID IN PHOSPHOLIPID	PER CENT PHOSPHOLIPID FATTY ACID				IODINE NUMBERS			
		Number of determinations	Mean	Standard deviation	Range	Number of determinations	Mean	Standard deviation	Range
214	63	3	2.64	0.57	2.65–2.75	4	32	7.6	30–35
214PA5	68	3	3.94	0.26	3.80–4.07	2	36	2.0	34–38
225	67	3	2.72	0.14	2.68–2.78	3	67	3.5	64–68
229B	64	3	3.21	0.17	3.18–3.28	3	33	5.4	31–37
234	64	4	2.90	0.24	2.69–3.08	3	60	6.3	52–65
239-1	73	3	3.18	0.10	2.97–3.37	4	47	3.5	43–50
248-1	67	4	3.27	0.01	3.13–3.42	3	77	3.2	76–77
300-1	65	3	3.26	0.14	3.22–3.34	3	50	6.5	47–54
657	61	3	2.36	0.01	2.31–2.44	3	86	3.6	83–91

fairly constant in amount and normal when compared to the values for all the known phospholipids.

The amount of phospholipid fatty acids in the dried bacteria is approximately 3 per cent. The iodine numbers also indicate here that the amount of unsaturated acid present is small. If one compares the iodine number of the phospholipid with that of the phospholipid fatty acid, it will be noted that with bacterial strains 214, 214PA5, 248-1, 300-1, the iodine number of the phospholipid is higher than that of the phospholipid fatty acid. Theoretically this is not possible. It has been observed by other workers, however, and has been explained by the hypothesis

that some oxidation of the unsaturated fatty acids takes place on separating the fatty acids from the phospholipid molecule.

Total lipid. The total lipid is a calculated value arrived at by adding the values for the neutral fat, the phospholipids, and the non-saponifiable portion of the alcohol-ether extract. By subtracting the value for the phospholipid fatty acids from the value for total fatty acid, the percentage of fatty acid present in the organisms as neutral fat is obtained. The non-saponifiable portion usually contains the sterol fraction. These values are shown in table 4.

TABLE 4

Analysis of bacterial lipids. Total lipid, non-saponifiable matter and neutral fat fatty acids based on dry weight

ORGANISM	CALCULATED AVERAGE TOTAL LIPID	CALCULATED AVERAGE NEUTRAL FAT FATTY ACIDS	NON-SAPONIFIABLE MATTER*
214	7.36	1.57	1.43
214PA5	7.91	0.43	1.82
225	4.45	0.00	0.38
229B	5.27	0.00	0.23
234	6.88	1.68	0.72
239-1	4.66	0.00	0.31
248-1	6.44	1.14	0.41
300-1	5.44	0.17	0.40
657	5.36	0.56	0.40

* Based on one determination only.

The total lipid in these organisms varies from approximately 4.5 to 8.0 per cent, these variations showing no correlation with the species or type. The amount of neutral-fat fatty acids also shows uncorrelated variations and ranges from zero to almost 2 per cent. The apparent lack of neutral-fat fatty acids in some cases indicates that the only fatty acids present are in the phospholipid.

The non-saponifiable portion of the lipid represents, for the most part, a very small fraction. Only strain 214 and its substrain 214PA5 show appreciable amounts. Just what this non-saponifiable portion represents is not known, since the amount available was not sufficient to permit further tests to be made

upon it. Ordinarily this portion would represent the sterol fraction of the total lipid from blood, tissues, *etc.*, but the organisms investigated contained no measurable amounts of any sterol.

Phosphorus and nitrogen in the phospholipid. To determine whether the phospholipids of the enteric bacilli are similar to those commonly found in animal and plant tissues, phosphorus and nitrogen determinations were made. Such analyses indicate the type of phospholipid concerned. The amount of phosphorus in ordinary lecithin and cephalin ranges from 3.75 to 4.25 per cent, depending upon the kind of fatty acid present in the molecule, while the nitrogen content ranges from 1.72 to 1.92 per

TABLE 5

Analysis of bacterial phospholipids. Phospholipid phosphorus and nitrogen

ORGANISM	AVERAGE PER CENT OF PHOSPHOLIPID		ATOMIC PHOSPHORUS-NITROGEN RATIO
	Phosphorous	Nitrogen	
214	4.40	0.00	1:0
214PA5	2.77	1.84	1:1.3
225	3.41	2.89	1:1.8
229B	3.41	1.66	1:1.1
234	3.11	2.03	1:1.5
239-1	4.66	1.28	1:0.5
248-1	3.51	1.74	1:1.1
300-1	3.45	2.33	1:1.5
657	3.68	1.40	1:0.9

cent. Chibnall and Channon (1927) described another type of phospholipid present in the leaves of the cabbage plant, and named it phosphatidic acid. It differs from the ordinary phospholipid in that it contains no nitrogen base, calcium or magnesium replacing this substance in the molecule. The percentage of phosphorus and nitrogen and the ratio of one to the other, therefore, aid in interpreting the type of phospholipid present.

The results of the analyses for phosphorus and nitrogen in the phospholipids of the bacteria are presented in table 5. The amount of phosphorus in the phospholipid is low, except for strains 214 and 239-1, in which it is higher than normal. The nitrogen values vary within the group some being higher than

usual and others lower. The results obtained with strain 214 are interesting since it is the only one that gave no yield of nitrogen in the phospholipid. When nitrogen is lacking, the percentage of phosphorus in the molecule is higher. The value for phosphorus in strain 214 agrees with this. A phosphatidic acid containing calcium in the molecule would theoretically have 4.48 per cent of phosphorus and no nitrogen. The phosphorus and nitrogen values of strain 214 coincide with those of such a phosphatidic acid. The phosphorus value for strain 239-1 is high, but the nitrogen content is normal, hence a phosphatidic acid is probably absent or is present only in small amounts.

The atomic ratio of phosphorus to nitrogen, with the exception of cultures 214 and 239-1, is approximately 1:1. Most of the values are slightly higher than this, probably because of some extraneous nitrogen. It has been found by MacLachlan (1935) and others that unless the phospholipid is carefully purified by dissolving it in water and precipitating it with acetone, the per cent of nitrogen will always be higher than the calculated theoretical values. Since there is a considerable loss of phospholipid in this procedure, the amounts of phospholipid available for the present study did not warrant its use. Nevertheless, the evidence is fairly conclusive that the phospholipids found in these organisms resemble those of animal and plant tissues with respect to the content of nitrogen and phosphorus.

No evidence of cholesterol or any other sterol in the lipids of the bacteria was found, *i.e.*, it was calculated to be present in amounts less than 0.3 per cent of the dry weight of the bacteria.

DISCUSSION

Although the total lipid of certain enteric bacilli is approximately what one might expect with other organisms, these bacteria are peculiar in two respects. First, the greatest portion of the total lipid is phospholipid. The significance of this finding is not clear. Possibly the phosphorus metabolism of the organisms is linked with the lipid metabolism, but to date there is no evidence to support such a view. In fact, the reports in the

literature seem to indicate that the phosphorus in the phospholipid represents only a small fraction of the total phosphorus in the bacterial cell. The second peculiarity is the absence of sterol. This absence has been observed in the acid-fast bacilli by Anderson *et al.* (1935). These compounds are apparently not synthesized in the organism in detectable amounts or they are rapidly broken down and thus are not detectable because they are not stored in the organism.

The differences between strains 214 and 214PA5 offer an interesting problem since the only difference between these two organisms is that the latter was grown for several serial passages on lithium-chloride agar. The analyses of these two cultures agreed quite well with respect to the total fatty acid content, but there was no agreement in the iodine numbers of their fatty acids. Of all the strains studied, the 214PA5 strain had the highest phospholipid content. Again, the iodine numbers of the phospholipid from the substrain 214PA5 and the parent strain 214 did not agree. The same situation existed in the case of the phospholipid fatty acids and the corresponding iodine numbers. The neutral fat fatty acids were much higher in strain 214 than in 214PA5. The most striking difference which occurred, however, was in the nitrogen content of the phospholipid of these strains. Whereas 214PA5 had a normal complement of nitrogen in the phospholipid, 214, the parent strain, had a phospholipid which was nitrogen free. It hardly seems reasonable that the serial passage on lithium-chloride agar could have induced such marked differences in the metabolism of the organisms, particularly when one realizes that this strain had been serially transferred on plain agar for over a year.

SUMMARY

1. An analysis of the lipids of nine strains of enteric bacilli has been made. The total lipid content of the organisms varied from 4.3 to 7.9 per cent of the dry weight. No cholesterol or other sterols were found.

2. The total fatty acid content varied from 2.5 to 4.6 per cent of the dried bacteria. The iodine numbers were low, varying from 42 to 82.

3. Phospholipids constituted the major portion of the total lipids. With one exception they represented about 60 per cent of the total lipid.

4. The fatty acid in the phospholipid molecule ranged from 63 to 73 per cent. As indicated by their iodine numbers, the fatty acids were not all alike.

5. The content of phosphorus and nitrogen of the phospholipid was normal, with the exception of a single strain which showed evidence of a phosphatidic acid in place of a phospholipid.

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THE CULTURAL AND ANTIGENIC PROPERTIES OF SHIGELLA SONNEI

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Received for publication August 19, 1938

The present study aims to define the taxonomic position of that group of dysentery bacteria commonly known as *Shigella paradysenteriae*, var. *sonnei* (Bergey, 1934). Most of the material on which this work is based has been recently isolated in this laboratory but a number of type strains of the species from The National Collection of Type Cultures in London, England, have been included for comparison. Also included for comparative purposes, are a number of strains of closely related members of the Genus *Shigella* which are mannitol-fermenters; namely, the groups known as Flexner, Dispar and Alkalescens.

ORIGIN OF STRAINS

A total of eighty-eight strains were studied. These included:

42 strains of *Shigella sonnei* isolated in this laboratory either within three months of the initiation of this work or while the work was in progress. All of these strains were recovered from the stools of patients with intestinal complaints;

12 strains of *Shigella sonnei* obtained from the National Type Collection at the Lister Institute, London;

2 strains of *Shigella alkalescens* obtained from Professor E. G. D. Murray both of which were original strains described by Sir Frederick Andrewes (1918);

3 strains of *Shigella dispar* isolated in this laboratory;

8 type strains of *Shigella dispar* obtained from the National Collection of Type Cultures at the Lister Institute, London.

4 strains of *Shigella dispar* obtained from Professor Murray

and originally described by him (1918) as his Class 4. At that time he identified them as the type described by Kruse (1907) as Type E, although he states (p. 387) "Class 4 is certainly equivalent to Kruse E. . . . The strains comprising Class 4 (Kruse E) would fall into the group comprised by Andrewes' *B. dispar*." Kruse E is at present generally held to be the same as *Shigella sonnei* (Gardner (1929) Gay (1935)).

5 strains of *Shigella paradysenteriae* (*flexner*) obtained from Professor Murray, representing the types V, W, X, Y and Z of Andrewes and Inman (1919) each of which is described in their monograph.

A. CULTURAL PROPERTIES

1. Colony form

The colony form was studied on plain agar, on Chandelier and on MacConkey plates. In general the Sonne strains produce colonies which are slightly larger, more granular and more opaque than those of the Flexner, Alkalescens and Dispar strains. All plates were kept in a moist jar for three weeks at 37°C.

a. Shigella sonnei colony. On original isolation all strains showed a uniform colony. At twenty-four hours incubation they were rather flat, slightly granular and opaque, about 3 to 4 mm. in diameter, with a smooth raised central zone grading out to a thin slightly irregular edge. The whole colony was lactose-negative. The colonies continued to increase in size and to spread peripherally until discarded and have reached three centimeters in diameter.

On further incubation, secondary colonies or papillae appeared on the surface of most, but not all, of the original colonies. These papillae, or daughter colonies, consisted of raised, smooth, entire, rounded outgrowths on the surface which, soon after their appearance, caused the medium immediately beneath them to show the lactose-positive change. These daughter colonies were usually 1 to 2 mm. in diameter within forty-eight hours after their appearance; some continued to increase in size while others did not. The length of time required for these papillae to appear varied from strain to strain but could be correlated roughly with

the time required for the individual strain to ferment lactose in peptone water medium.

Only one strain (out of fifty-four) failed to show secondary colonies. In this strain the centre of the colony became progressively more heaped up and irregular and eventually the entire colony went lactose-positive. Repeated platings of this strain failed to change its behavior.

Attempts were made to get these secondary papillae to breed true by carefully fishing them from the parent colony and replating through 10 to 12 generations. Each generation began as a lactose-negative colony upon which lactose-positive papillae appeared at about the same interval as on original isolation. The twelfth generation did not differ from the first generation even on a percentage basis. Similarly, the few original primary colonies which showed no lactose-positive papillae (although most other colonies of these same strains on the original plate did show numerous papillae) were replated up to twenty generations, alternating solid and fluid media, without changing the general appearance of any generation. The colonies always started as lactose-negative and most of them later developed lactose-positive papillae.

A second colony form appeared on plating out from older cultures or from cultures grown in 1:10,000 Brilliant-Green Peptone Water. This is a flat, entire colony with a bevelled edge, not granular and with a moist shiny surface, smaller than the ordinary colony, increasing little in size with further incubation and not developing papillae. These colonies do not breed true.

The two colony types gave identical fermentation reactions.

b. Shigella dispar colony. These strains showed colonies in every way similar to the granular type of the Sonnei colonies—including the formation of small, smooth secondary papillae which became lactose-positive.

c. Shigella alkalescens colony. These strains showed a somewhat smoother granular type of colony and they also later developed secondary papillae. These secondary papillae, however, always remained consistently lactose-negative.

d. Shigella paradysenteriae (Flexner's bacillus). Secondary papillae were less frequently encountered in the Flexner type. When they did appear they also remained consistently lactose-negative. The Flexner colony, in general, was the smoothest of all.

2. Microscopic appearance

Smears from the various colonies showed approximately the same morphology; all were gram-negative rods. The only difference noted was that the old strains seemed to contain more filamentous forms than freshly isolated strains.

3. Motility

Motility was checked on all strains by observing hanging drop preparations on eight- and on eighteen-hour cultures in beef-infusion broth at room temperature and at 37°C. In addition, each strain was tested for migration through semisolid agar at room temperature and at 37°C. All strains were uniformly non-motile.

4. Biochemical reactions

The reactions shown in table 1 were found to be the most useful in separating the groups. The sugar media consisted of 1 per cent of sugar in 1 per cent peptone water with Andrade indicator. The tubes were kept at 37°C. for sixty days. At first, two methods of controlling evaporation were used: either keeping the tubes filled to a mark with sterile distilled water, or waxing the cotton stoppers with paraffin. After twenty-three strains had shown identical results by both methods, the waxed plug method was adopted for the rest of the work in order to avoid the occasional contamination.

The litmus milk was kept in a tube with a waxed plug.

Indole was tested for by treating two-day and five-day growths in 1 per cent peptone water with Ehrlich's reagents.

Some of the type strains were put through the full examination only once, but most of the recently isolated strains were re-examined and the tests were repeated for all strains which showed

results not conforming with the general results. In these repeat tests, the only difference found was that two strains which failed to ferment lactose in the first sixty-day trial, did ferment lactose on retest in 27 and 32 days respectively.

As shown in table 1, both *Shigella sonnei* and *Shigella dispar* are sharply distinguished from *Shigella alkalescens* and *Shigella paradysenteriae* (flexner), and, incidentally, from all other *Shigella*, in the late fermentation of lactose. The Sonne group may be

TABLE 1

The most useful reactions for separating the mannitol-fermenting groups of dysentery bacteria

CLASSIFICATION	NUMBER OF STRAINS	GLUCOSE	SUCROSE	LACTOSE	MANNITOL	RHAMNOSE	XYLOSE	SORBITOL	DULCITOL	INDOLE	LITMUS MILK
Sonne	54	A	A 4–32 days	A 2–32 days	A	A	0 Very rarely A	0	0	0	Neutral to A about 2 days after Lactose
Dispar	15	A	A 2–19 days	A 1–6 days	A	A	A	A	0	+	Neutral to A about 2 to 8 days
Alkalescens	14	A	0	0	A	A 3–17 days	A	A	A	+	Neutral to very alkaline 3 to 10 days
Flexner	Compiled	A	var.	0	A	var.	0	var.	0	var.	var., A changing to alkaline

All strains non-motile, gelatin negative, salicin negative.

A = acid; 0 = no change during 60 days incubation; var. = variable, depending on the particular strain tested.

separated from the dispar group since the latter ferment xylose and sorbitol and produce indole.

Shigella alkalescens stands distinctly apart from the others in the production of alkalinity in litmus milk, a feature of diagnostic significance, with the fermentation of dulcitol.

The reactions of the Flexner group are not based on the five strains used in this study alone, but compiled from the literature as well as our own experience with other Flexner strains in the past.

In the fermentation of mannitol, all of these groups are related. As Murray (1918), Andrewes and Inman (1919), and Gardner (1929) point out, this is the one important fermentable substance of taxonomic value which has stood the test of time.

It is apparent from the data given in table I that complete identification of *Shigella sonnei* by cultural tests alone cannot be made within a time short enough to be of clinical value. The distinguishing changes in lactose and litmus milk often take two or three weeks to develop. Thus, for rapid diagnosis any mannitol-fermenting dysentery organism must be subjected to serological tests.

B. ANTIGENIC PROPERTIES

The serological properties of the groups were studied by the use of agglutination, agglutinogenic power and the agglutinin absorption reaction.

Methods

From an original plate the smoothest colony was selected and large agar plates inoculated. Incubation was for eighteen hours in order to avoid the occurrence of secondary papillae (lactose-positive variants) which, in our experience, never appeared under forty-eight hours. The growth was scraped from the surface of the plate with a spade and suspended in 0.85 per cent NaCl to which 0.3 per cent of formalin was added. Such suspensions remained stable, having no tendency to sediment spontaneously as "rough" strains would be expected to do. They were standardized by comparison with standard opacity tubes by Brown's method.

Rabbits were immunized by the intravenous injection of 500 million, 1000 million and 2000 million formalized bacteria on the first, sixth and eleventh days respectively. The animals were bled on the seventeenth day and the serum obtained usually reached a titre of between 5,000 and 40,000.

For agglutination tests, the antigen suspensions were standardized to a final concentration of 400 million bacteria in each tube, the total volume of fluid being 2.0 cc. The range of serum

dilutions in each test was from 1 in 40 to 1 in 80,000. Lower dilutions were avoided because experience had shown that normal rabbit serum often agglutinated various dysentery bacteria in lower dilution but that the titre rarely exceeded 1 in 40. The end-point selected in each test was the greatest dilution of serum which gave complete agglutination and a clear supernatant fluid after twenty-four hours incubation at 37°C.

For agglutinin absorption tests the antigens were standardized to a final concentration of 135,000 million bacteria per cc. of undiluted serum. Absorptions were done in a final 1-in-10 dilution of serum in a total volume of 10 cc; incubated for 5 hours at 37°C.; then left in the ice box overnight before centrifuging. The absorbed serum was then tested not only against the homologous strain but also against several others.

At the beginning of this study an agglutinating serum was prepared in rabbits for all of the strains available at that time. Twenty-seven serums in all were prepared against twenty-seven different strains. Of these, 14 were *S. sonnei*, 5 were *S. paradysenteriae* (flexner), 4 were *S. alkalescens* and 4 were *S. dispar*. Later, additional strains were added to the series, so that our results are finally based on tests involving twenty-seven serums and eighty-eight strains. 54 of these strains were finally classified as *S. sonnei*, 14 as *S. alkalescens*, 15 as *S. dispar* and 5 were type strains of *S. paradysenteriae* (flexner).

Table 2 shows the result of cross agglutinations between 14 serums and 22 strains of *S. sonnei*.

The existence of two groups seems indicated by the table; neglecting, for the moment, differences in agglutinability of strains—a factor not controlled in this type of experiment but capable of being checked by absorption tests.

The first group included 13 strains isolated in this laboratory and 3 National Type Collection strains. Nine other National Type Collection strains as well as 17 other strains isolated in this laboratory are not listed in the table but have been shown to fall into this group. Each strain is agglutinated to full titer by a serum produced from any other strain. This result is in agreement with that of many previous investigators who contend that

the sonnei group is antigenically homogeneous, (Kruse (1907), Hilgers (1920), Fraser, Kinlock & Smith (1926), Kerrin (1928), Nelson (1930) and Koser, Reiter, Bortniker and Swingle (1932)),

TABLE 2

Cross agglutinations of 22 strains of Shigella sonnei with rabbit agglutinating serums prepared from 14 of these strains

ANTIGEN	ANTISERUM													
	Nat. type col. 2318	Nat. type col. 2182	Nat. type col. 268	B593	CMH683	CMH218	B314	CMH212	CMH207	CMH232	A3252	B637	B217	B597
Nat. type col. 2318	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Nat. type col. 2182	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Nat. type col. 268	10	10	10	10	10	10	10	10	10	10	10	10	10	10
B593	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH683	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH218	10	10	10	10	10	10	10	10	10	10	10	10	10	10
B314	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH212	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH207	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH232	10	10	10	10	10	10	10	10	10	10	10	10	10	10
A3252	10	10	10	10	10	10	10	10	10	10	10	10	10	10
B637	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH8122-b	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH8187-1b	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH8187-2a	10	10	10	10	10	10	10	10	10	10	10	10	10	10
CMH8222-1b	10	10	10	10	10	10	10	10	10	10	10	10	10	10
B217	5	3	2	4	2	3	2	2	2	2	3	2	10	10
B597	4	2	2	4	2	3	2	2	2	2	3	2	10	10
CMH8122-a	5	2	3	3	2	3	3	2	2	3	3	2	10	10
CMH8187-1a	3	3	2	4	3	2	2	2	2	2	2	2	10	10
CMH8207	2	2	2	2	3	2	2	2	2	2	2	2	10	10
CMH8222-1a	5	3	2	4	2	3	2	2	2	2	3	2	10	10

Numbers refer to the end point tube (complete agglutination) in a series where 10 represents the titre for the homologous strain.

though others (Sonne (1915), Murakami (1927), Leuchs and Plockmann (1927)) suggest the existence of two serological varieties. Since it is apparently the most common type of the sonnei organism isolated, we propose calling this group *Shigella sonnei*, Type I.

The second group includes 6 strains isolated in this laboratory. An additional 6 strains, also isolated here but not listed in the table, fall into this group as well. These strains are agglutinated to not more than a small fraction of the titre by any serum of Type I. However, a serum prepared from two of these strains, B217 and B597, will agglutinate all strains of both types to full titre. Since this group is less commonly found than the former we propose calling it *Shigella sonnei*, Type II. It should be recorded that this Type II is probably more distinctive of the species *Shigella sonnei* than Type I as judged by taxonomic standards—a point which will be further supported by later evidence.

In order to check these results and to control the factor of variation in agglutinability of strains, absorption tests were carried out.

Table 3 shows the result of some of these absorptions. The table is condensed from a large series of absorptions in which the results illustrated were characteristic of the entire series.

These absorptions confirm the results of straight agglutination tests as to the existence of two types of *Shigella sonnei*. They also warrant additional statements regarding the antigenic structure of the two types.

Type II evidently contains both Type I and Type II antigens in considerable amounts because, not only will its serum agglutinate both types to full titre, but it will itself completely absorb the agglutinins from serum of both types.

Type I, on the other hand, apparently contains the Type I antigen in predominance, with a relatively small amount of Type II antigen, since its serum will agglutinate Type II to only a fraction of its titre, and it will absorb not more than 50 to 75 per cent of the agglutinins from a Type II serum.

The question might arise whether we are dealing here with a smooth-rough type of variation such as Johnston and Kaake (1932) concluded was the explanation of the two types described by Johnston and Brown (1930). In its original definition the smooth-rough variation was related to differences in colony form, kind of growth in broth and salt sensitiveness and more recently

to loss of an antigenic component. Although the Sonne colony is never quite as smooth as the Flexner colony even on primary isolation it grows with uniform turbidity in broth, does not sediment spontaneously in saline and the two colony forms we describe are not related to the immunological types by agglutination.

TABLE 3

Reciprocal absorptions between two Type I strains of Shigella sonnei (nat. type col. 2318 and B593) and two Type II strains (B217 and B597)

	SERUM	ABSORBING STRAIN	ABSORBING DOSE OF UNDILUTED SERUM	TITRE FOR HOMOLOGOUS STRAIN		REDUCTION OF HOMOLOGOUS TITRE
				Unabsorbed	After absorption	
			millions per cc.			*per cent*
Homologous absorptions Type I	N.T.C.2318	B593	135,000	5,120	0	100
	N.T.C.2318	N.T.C.2318	135,000	5,120	0	100
	B593	N.T.C.2318	135,000	40,000	640	99
	B593	B593	135,000	40,000	80	99
Heterologous absorptions	N.T.C.2318	B217	135,000	5,120	0	100
	N.T.C.2318	B597	135,000	5,120	0	100
	B593	B217	135,000	40,000	1,280	96
	B593	B597	135,000	40,000	640	99
	B217	N.T.C.2318	135,000	2,500	1,280	50
	B217	B593	135,000	2,500	1,280	50
	B597	N.T.C.2318	135,000	2,500	640	75
	B597	B593	135,000	2,500	1,280	50
Homologous absorptions Type II	B217	B597	135,000	2,500	0	100
	B597	B217	135,000	2,500	0	100

Referring to the clinical records we find that in some instances different colonies of the same original stool plate fall into different serological types. Thus, in case CMH 8122, colony *a* is Type II while colony *b* is Type I. In case CMH 8187, colony *1a* is Type II while colonies *1b* and *2a* are Type I. In case CMH 8222 colony *1a* is Type II while colony *1b* is Type I. These observations might suggest that we are dealing with a *diphasic* variation which has hitherto been considered to apply only to flagellated species of bacteria. (See however, Takita (1937).)

Our data at present do not permit a decision on these points and to pursue them further would unnecessarily prolong this paper and would deviate from our original purpose.

Tentatively, we suggest that *Shigella sonnei* contains two predominant antigens which we call Type I and Type II. The Type II is the more distinctive Sonne bacillus in that it contains a considerable amount of both antigens while the Type I seems to lack some antigen present in Type II. In other words, an anti-Type II serum will agglutinate all types of Sonne strains that we have encountered but an anti-Type I serum will fail to agglutinate some of them except in fractions of the serum titre.

Having determined the major antigens in *Shigella sonnei* we next studied the minor antigens which relate it to the other mannitol-fermenting groups of dysentery bacteria.

Table 4 is condensed from a larger series to illustrate these relationships.

It is apparent from this table that *Shigella dispar* is itself antigenically heterogeneous; this confirms the observations of Forsyth (1933) and of Watanabe (1935). The Flexner antigens (*Shigella paradysenteriae*) are omitted from the table since it is well known that they are heterogeneous. *Shigella alkalescens* appears to be homogeneous and it may be remarked that 10 additional strains not listed in the table were also agglutinated to full titre by the four Alkalescens serums.

The Sonne Type I antigen is agglutinated to a slight extent by Flexner, Alkalescens and Dispar serums. It is a curious fact that, in a larger series comprising 42 strains of Sonne, every one showed some agglutination with Flexner V, W, X and Z serums but uniformly failed to be agglutinated by a Flexner Y serum. In contrast to this the Alkalescens antigens were uniformly agglutinated by V, W, X and Y serums and failed to be agglutinated by a Flexner Z serum.

The Sonne Type II antigens showed less obvious relation to either Flexner, Alkalescens or Dispar. Of the two antigens illustrated, B597 appears to be the more nearly a pure *Shigella sonnei* antigen in that it fails to react with any of these serums while B217 reacts with both Flexner Z and Alkalescens serums—a property of this strain not disclosed by any of our previous tests.

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TABLE 4

Cross agglutination of representative strains of Shigella sonnei Type I and Type II, Shigella alkalescens and Shigella dispar with Flexner, Alkalescens, Dispar and Sonne serums

	ANTIGEN	FLEXNER					ALKALESCENS				DISPAR				SONNE	
		Antiserum														
		"V" Lister	"W" D.J.	"X" Toner	"Y" Ledingham	"Z" Whittington	Alkalescens "Andrewes"	B598	B599	B1374	Nat. type col. 1602	Nat. type col. 4645	Nat. type col. 4168	"E" Ledingham (Murray class 4)	Nat. type col. 2318 (Type I)	B597 (Type II)
Shigella sonnei Type I	Nat. type col. 2318	2	1	2	0	4	5	4	4	5	3	6	2	2	10	10
	Nat. type col. 2182	2	2	2	0	4	5	4	4	5	2	4	2	2	10	10
	Nat. type col. 268	2	2	2	0	5	5	4	4	5	3	4	2	2	10	10
	B593	3	4	3	0	5	5	4	4	5	3	4	2	2	10	10
	CMH683	2	3	2	0	5	5	4	4	5	2	3	2	2	10	10
	CMH218	2	2	2	0	5	5	4	4	5	3	2	2	2	10	10
Shigella sonnei Type II	B217	0	0	0	0	2	5	2	3	5	0	0	0	0	5	10
	B597	0	0	0	0	0	0	0	0	0	0	0	0	0	4	10
Shigella alkalescens	Alkalescens "Andrewes"	6	4	4	4	0	10	10	10	10	6	5	0	0	0	0
	B598	6	5	4	5	0	10	10	10	10	5	5	0	0	0	0
	B599	6	5	4	5	0	10	10	10	10	6	4	0	0	0	0
	B1374	3	3	4	4	0	10	10	10	10	5	4	0	0	0	0
Shigella dispar	Nat. type col. 1602			3	2		0				10	0	10	10	0	3
	Nat. type col. 4645			4	2		2				0	10	0	0	0	0
	Nat. type col. 4168			4	3		0				10	8	10	10	0	2
	"E" Ledingham (Murray class 4)			4	2		5				10	0	10	10	0	0
	19A (Murray class 4)			4	2		2				10	0	10	10	0	0
	67B (Murray class 4)			4	2		3				10	0	10	10	0	0
	86A (Murray class 4)			4	2		2				10	0	10	10	0	0
	A3034			4	2		1				0	10	0	0	0	0

Numbers refer to the end point tube (complete agglutination) in a series where 10 represents the titre for the homologous strain. Blank spaces mean that the test was not carried out.

The results of table 4 again confirm our views that the Type II Sonne bacillus is the more typical of the species in that its antigens are more redominantly *Shigella sonnei* in character. If it contains the antigens common to the Flexner, Alkalescens and Dispar groups, these are not brought into evidence by this type of test.

Reciprocal absorption tests failed to add to the information given in table 4.

DISCUSSION

If the dysentery group as a whole be divided primarily on a basis of mannitol fermentation we find that the non-mannitol-fermenters, *Shigella dysenteriae*, *Shigella ambigua*, (Schmitz bacillus) stand sharply apart from the others by reason of a number of properties; cultural, antigenic and pathogenic. The next subdivision, among the mannitol fermenters themselves, is not so clear cut and overlapping occurs in many of the properties of each species. The most striking and most reliable feature which can be used to separate the Sonnei-Dispar group from the Flexner group is the fermentation of lactose, but this may be delayed as long as three or four weeks. Because the individual strains of the Flexner group are so variable in themselves it is impossible to distinguish between Flexner and Sonne or Dispar on cultural grounds alone until sufficient time has been allowed for the fermentation of lactose. Serologically also, enough overlapping occurs to require a complete series of agglutinations to the full titer of the serum before reliance can be placed on this type of information.

It is, of course, just this similarity of properties among the mannitol-fermenting dysentery organisms that justifies considering them more closely related to one another than is the relationship of any one of them to either Shiga or Schmitz.

We believe that Alkalescens belongs in the mannitol-fermenting group of dysentery organisms in spite of its indefinite pathogenicity. From a taxonomic viewpoint, pathogenicity must be regarded as only one of the properties of a species, not necessarily more important *per se* than a combination of other proper-

Numbers refer to the end point tube (complete agglutination) in a series where 10 represents the titre for the homologous strain. Blank spaces mean that the test was not carried out.

ties. For similar reasons we have excluded from our study such species as have been described under the name of "Coli Anaerogenes." On closer examination some of these strains are either true Sonne or Dispar or they produce small quantities of gas, often late, and thus appear to be more nearly related to the Escherichiae (Coli-Aerogenes group) than to the dysentery group. (Topley and Wilson 1936)).

The occurrence of secondary papillae on colonies is not confined to members of the Genus *Shigella*; it is occasionally found also among other genera in *Eberthella* and *Salmonella*. But in the Genus *Shigella* it reaches a marked development. Less commonly seen in the Shiga and Schmitz species, it becomes more frequent in the Flexner and Alkalescens species and is the rule rather than the exception in Sonne and Dispar. So much is this so, that the phenomenon of late lactose fermentation in Sonne and Dispar seems dependent on the production of these papillae. They are, in fact, variants of the original strains with new fermenting powers. We have consistently failed to get the variants to breed true; they also revert to the lactose-negative state on fresh media and the fermentation of this sugar occurs only in ageing cultures. The failure to secure a pure culture of lactose-positive Sonne has prevented our using it for antigenic analysis.

While we feel certain of the presence of at least two major antigens in *Shigella sonnei*, we fine no indication that they are associated with a rough-smooth variation. If "roughness" be defined as a rough colony which does not remain in suspension in the presence of electrolytes, these antigens are both smooth antigens. If, on the other hand, "roughness" is defined as the quantitative loss of some antigenic component, we find that our Type II possesses an antigenic component not present in our Type I; but both serological types are independent of the variation observed in colony form. We have not investigated the possibility of a diphasic variation.

From the standpoint of practical diagnosis only the Type II antibody can be relied on to detect all strains of *Shigella sonnei*; the Type II antibody will agglutinate all strains to full titre while the Type I antibody will agglutinate only the Type I

antigen to full titre and the type II antigen to a fraction of its titer.

The minor antigens of *Shigella sonnei* which show cross reactions with *Shigella paradysenteriae*, *Shigella alkalescens* and *Shigella dispar* again emphasize the close taxonomic relationship of these four species. It is interesting that Type II, which is regarded as the more distinctive, shows fewer cross reactions with the other three than does Type I.

We have searched for, and failed to find, any cultural or biochemical property by which Type I strains might be distinguished from Type II strains. All of our Type II strains have been recent isolations but an even greater number of recent strains belong to Type I. Indeed, both types have occurred at the same time on the same original plate from the same stool specimen.

SUMMARY AND CONCLUSIONS

1. *Shigella sonnei* is closely related culturally and antigenically to three other members of the mannitol-fermenting species of dysentery organisms; namely the *Shigella paradysenteriae* (Flexner's bacillus), *Shigella alkalescens* and *Shigella dispar*.

2. The most outstanding distinguishing property of *Shigella sonnei*, the late fermentation of lactose, is due to the appearance in ageing cultures of variants with new fermenting powers. These variants are seen in the original colony as secondary papillae arising directly from the mother colony only after the colony has aged.

3. These variants are not permanent. When transferred to fresh medium, they immediately revert to the lactose-negative state and, eventually, these transfers in turn produce lactose positive variants. This succession of events may be continued indefinitely.

4. At least two major antigens are present in *Shigella sonnei* both of which are present in all strains but to varying degrees. *Shigella sonnei* Type II is the more representative type since both antigens are abundantly represented in it; but it is least frequently encountered. *Shigella sonnei* Type I is most fre-

quently found and contains mainly Type I antigen with a relatively small amount of Type II antigen.

5. Minor antigens in *Shigella sonnei* are common to *Shigella dispar, Shigella alkalescens, Shigella paradysenteriae.*

6. On a basis of cultural and antigenic properties the Sonne group differs sufficiently from the closely related Flexner, Dispar and Alkalescens groups to warrant recognition as a separate species of the Genus *Shigella* rather than as a variant of *Shigella paradysenteriae.* This confirms the species *Shigella sonnei* (Weldin).

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THE ABSORPTION OF BACTERIOPHAGE BY SENSITIZED ENTEROCOCCI

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Received for publication August 20, 1938

It is generally accepted that the first step that takes place in the lysis of bacteria by a bacteriophage consists in the absorption of the bacteriophage particle to the surface of the organism, and that organisms that are phage-resistant will not absorb bacteriophage. Exceptions to this principle, namely where a non-susceptible organism is capable of binding phage to a high degree, have been encountered by Levine, Frisch, and Cohen (1934), Burnet (1934) ,Rakieten *et al.* (1936), and Rakieten and Tiffany (1938). This preliminary absorption by the bacterial cell is in all probability due to some element of the complex antigenic structure of the organism. Burnet, in a series of investigations dealing with bacteria belonging to the dysentery and salmonella groups and their appropriate phages, has shown that a marked parallelism exists between antigenic reactions and phage susceptibility, and has brought forth evidence pointing to the heat-stable somatic or O-antigen as the fraction which specifically absorbs phage. *Salmonella* strains having a minor O-antigen in common with *Salmonella enteritidis* showed a degree of sensitivity to some of the enteritidis phages, whereas this sensitivity was, with one exception, lacking among organisms not possessing an O-antigen in common with *S. enteritidis* (Burnet, 1927; Burnet and McKie, 1930; Burnet, 1930, 1932, 1934; Gough and Burnet, 1934). Thus, the evidence that the phage-binding portion of the bacterial cell is linked with that portion of the cell that binds the O-agglutinin appears to be highly convincing.

If the component of the bacterial cell capable of binding phage is the agglutinogen, then, when this antigen is united with spe-

cific agglutinin, phage absorption by these cells should be negligible, as compared with the degree of positive absorption by bacterial cells whose heat-stable surface antigen has been untouched by specific sera. In order to test this hypothesis a number of experiments were carried out. The results that were obtained confirmed the above hypothesis, namely that there is a direct relation between "covering" of agglutinogen and lack of absorption of phage. At the same time numerous attempts were made to prove that the agglomeration of these cells was not in itself the reason for their failure to fix phage. The present report deals with the experimental data obtained to substantiate our conclusions.

The organisms used were three members of the enterococcus group, strains Z-1, Z-11, and E-16. Cultures Z-1 and Z-11 belong to the species *Streptococcus liquefaciens* according to Sherman's classification (Sherman, 1937), and had originally been isolated from the blood of patients with sub-acute bacterial endocarditis. Strain E-16 was isolated from a case of ulcerative colitis, and was typical of *Streptococcus fecalis*. These organisms, together with other members of the group have been used in a previous investigation and are described more completely in a communication recently published (Rakieten and Tiffany, 1938).

The sera were prepared by repeated intravenous inoculation in rabbits of formalin-killed vaccines of the appropriate organisms and were preserved in 1:10,000 merthiolate. Serum 84 was prepared against culture Z-1; serum 11 against culture Z-11; and serum 87 against strain E-16. All of these cultures were related closely serologically, but were not identical as may be seen by observing tables 1a and 1b, in which the agglutination and agglutinin absorption titers are presented.

In table 1a the figures represent the highest dilution at which agglutination was unequivocal, although not necessarily complete. The italic figures represent the titers obtained with homologous systems. In table 1b the figures showing the titer of serum with homologous organism after absorption by an heterologous organism are italic. From this last table it is clear that there is no complete reciprocal absorption and that no one

of the three organisms is serologically identical with another, although they do have some degree of antigenic similarity. The only system in which there is important absorption of the homologous or "major" agglutinin of a serum by a heterologous organism is that in which serum 11 is absorbed with culture E-16. Strain Z-11 on the other hand leaves untouched the "major" agglutinin in serum 87. Thus, these two organisms, Z-11 and E-16, while not identical, are more closely related to one another than is either to culture Z-1. Such one-sided relationships be-

TABLE 1a

Serum titers for homologous and heterologous organisms before absorption

	SERUM 84 (Z-1)	SERUM 11 (Z-11)	SERUM 87 (E-16)
Tested vs. strain Z-1	*5000*	2560	2560
Tested vs. strain Z-11	2560	*2560*	5000
Tested vs. strain E-16	2560	1280	*5000*

TABLE 1b

Relationship of strains as shown by reciprocal absorptions

	SERUM 84 (Z-1)	SERUM 11 (Z-11)	SERUM 84 (Z-1)	SERUM 87 (E-16)	SERUM 11 (Z-11)	SERUM 87 (E-16)
	Absorbed with culture					
	Z-11	Z-1	E-16	Z-1	E-16	Z-11
Tested vs. strain Z-1	*5000*	80	*1280*	<20		
Tested vs. strain Z-11	80	*1280*			<40	320
Tested vs. strain E-16			160	*5000*	80	*5000*

tween organisms are known, and are described in some detail by Krumwiede, Cooper, and Provost (1925). These authors state that "between the two degrees of a complete reciprocal reaction and an absence of such reaction, all degrees of partial reciprocal reactions may be encountered." None of these sera contained antibodies specific for Lancefield's Group D streptococcus.

The bacteriophage employed is polyvalent for many strains of enterococci and was propagated against these cultures many times in Savita broth (Rakieten, 1932). This phage is com-

pletely absorbed by heat-killed cultures of Z-1, Z-11, and E-16, as well as by watery extracts prepared from these same strains. Unless stated otherwise, the suspending medium used in this study was Savita broth.

EXPERIMENTAL

The method used to demonstrate the blocking effect of immune antibodies upon the so-called phage receptors of the bacterial cells was as follows. Standard suspensions of cultures which had been killed by exposure in sealed ampoules to a temperature of 70°C. for thirty minutes, were suspended in broth containing known dilutions of anti-serum. After an incubation period of from one to three hours in a water bath at 37°C., the degree of agglutination was determined microscopically; following this, a constant volume of bacteriophage was added to each tube and the entire series incubated for twenty hours at 35°C. The amount of bacteriophage added to each tube was such that, providing no absorption took place, a countable number of plaques was produced. 0.02 cc. from each tube was then layered over a segment of an agar plate that had previously been inoculated with a fresh susceptible culture of Z-1. Throughout this work culture Z-1 has been used to demonstrate plaque production, since it was found that the results were approximately the same regardless of which organism was employed as the indicator. The tubes were again checked for evidence of aggluination by the use of the microscope. Plaque counts were done after an appropriate incubation of the agar plates. The degree of agglutination was thus known and recorded by one of us long before the plaque counts were made by the other. Throughout the entire study the individual responsible for adding phage and carrying the experiment to completion did not know the contents of the tubes containing the various mixtures. Proper controls were always run with each new series of experiments.

Experiment 1. The phage-absorbing power of a heavy suspension of organisms in the presence of homologous anti-serum

Heat killed suspensions of culture Z-1 in broth were set up in a series of tubes containing different dilutions of homologous im-

mune serum as well as normal rabbit serum. The total volume in each tube was 5 cc., the concentration of organisms was four billion per cubic centimeter. After a preliminary incubation period of three hours, a constant quantity of bacteriophage was added to each tube. Following an incubation period of twenty hours, the tubes were examined for agglutination and portions removed and tested for their ability to produce plaques. Table 2 illustrates the results obtained.

The results set forth in this table indicate that the antibodies present, while capable of agglutinating the bacteria, did not,

TABLE 2

TUBE NUMBER	ORGANISM SUSPENSION	SERUM DILUTION (BP. ADDED)	AGGLUTINATION	PLAQUE COUNT
1	Heat killed Z-1	IS 84, 1:50	4+	0
2	Heat killed Z-1	IS 84, 1:160	4+	0
3	Heat killed Z-1	IS 84, 1:2000	±	0
4	Heat killed Z-1	NRS, 1:50	−	0
5	Heat killed Z-1	NRS, 1:160	−	0
6	Heat killed Z-1	NRS, 1:2000	−	0
7	None	IS 84, 1:50		cfl
8	None	IS 84, 1:160		cfl
9	None	IS 84, 1:2000		cfl
10	Heat killed Z-1	None		0
11	None	Savita broth. None		cfl

IS = immune rabbit serum; NRS = normal rabbit serum; cfl = plaques confluent (over 500); 4+ = complete agglutination, macroscopically, but microscopic examination revealed many unagglutinated organisms.

under the conditions of this experiment, in any way interfere with the complete absorption of the added bacteriophage. In this heavy suspension, however, microscopic examination revealed free and unagglutinated cells in such quantitities that, conceivably in the absence of complete coating by the antibodies, they alone could be responsible for the fixation of the bacteriophage. It is of interest to note that in no instance did any of the dilutions of serum inhibit the phage. These findings, which were obtained many times in repeating similar experiments, suggest that any interference with phage absorption by surface antigen in the presence of immune serum might be demonstrable only after a careful adjustment of the various elements employed in the test.

Experiment 2. The titration of reagents

An effort was made to determine how far one could dilute the standard suspension of bacteria and still get significant absorption of the added phage. A heat-killed suspension of culture Z-1 (four billion per cubic centimeter) was prepared in the usual manner and serial dilutions set up in broth, the final volume being 5 cc. Constant quantities of diluted bacteriophage were then added and the tubes incubated for twenty hours at 35°C. 0.02 cc. from each tube was then layered over a segment of an agar plate that had previously been streaked with culture Z-1.

TABLE 3

TUBE NUMBER	SUSPENSION (HEAT-KILLED Z-1) 4 BILLION PER CC., PLUS PHAGE	PLAQUE COUNT
1	Undiluted	0
2	Diluted 1:2	0
3	Diluted 1:4	0
4	Diluted 1:8	0
5	Diluted 1:16	0
6	Diluted 1:32	0
7	Diluted 1:64	0
8	Diluted 1:128	0
9	Diluted 1:256	4
10	Diluted 1:512	13
11	Savita broth	500

After incubation the plates were examined for the presence of plaques and counted. The results are listed in table 3.

The turbidity of the suspension decreased uniformly to tubes 8 and 9, in which cloudiness was barely perceptible. Tube 10 in any light appeared as clear as the control without organisms (tube 11). Tubes 8, 9 and 10, when examined microscopically by hanging drop and smear, showed very few organisms (the calculated number of organisms in tube 10 is less than 8,000,000 per cubic centimeter). These results suggested that in the previous experiment (table 2) suspensions far too heavy may have been employed, since a suspension having the same density and diluted 512 times is still capable of inactivating phage.

Therefore, in the second part of this experiment a standard

suspension of heat-killed culture of Z-1 of nearly the same density (3.8 billion per cubic centimeter) was made and serial dilutions prepared. To each dilution a constant amount of immune and normal rabbit serum was added, and, following an hours incubation, 0.1 cc. of bacteriophage was inoculated into each tube. After remaining at 35°C. for twenty hours a portion from each

TABLE 4

TUBE NUMBER	SUSPENSION (HEAT-KILLED Z-1) 3.8 BILLION PER CC.	SERUM DILUTION (BP. ADDED)	AGGLUTINATION	PLAQUE COUNT
1	Undiluted	IS 84, 1:50	+	0
2	Undiluted	NRS, 1:50	±	0
3	Undiluted*		−	0
4	Diluted 1:4	IS 84, 1:50	+	1
5	Diluted 1:4	NRS, 1:50	−	0
6	Diluted 1:4*		−	0
7	Diluted 1:16	IS 84, 1:50	+	43
8	Diluted 1:16	NRS, 1:50	−	0
9	Diluted 1:16*		−	9
10	Diluted 1:64	IS 84, 1:50	±	296
11	Diluted 1:64	NRS, 1:50	−	6
12	Diluted 1:64*		−	0
13	Diluted 1:128	IS 84, 1:50	−	314
14	Diluted 1:128	NRS, 1:50	−	43
15	Diluted 1:128*		−	0
16	Diluted 1:512	IS 84, 1:50	−	380
17	Diluted 1:512	NRS, 1:50	−	380
18	Diluted 1:512*		−	5
19	None*	IS 84, 1:50		379
20	None*	NRS, 1:50		450
21	None*	None		450

* Broth.

tube was removed (0.02 cc.) and tested for its ability to produce plaques. The results are indicated in table 4.

With a heavy suspension of organisms (undiluted, to a dilution of 1:16) the immune serum in the dilution used is unable to affect demonstrably the phage-absorbing power of these cells. When the number of bacteria is progressively decreased, and these cells are allowed to come in contact with the same dilution of immune serum, the surface antigens of these cells do appear to

be completely "covered" by the antibodies, for when phage is added to these cells there is no significant reduction in corpuscular count. It is important to note that while, in general, the normal rabbit serum has little or no effect on these same cell receptors and therefore phage is absorbed, that in certain of the bacterial dilutions normal rabbit serum does seem to be able to cover surface antigen so that it is not free to absorb bacteriophage, (tubes 14 and 17). The normal rabbit serum used showed a definite agglutination of culture Z-1 when the serum was diluted 1:40, and a slight amount of agglutination in a dilution of 1:80. It is quite likely, therefore, with the small number of organisms present in tube 17, that the antibody concentration in the 1:50 dilution of normal serum was not without effect, and combined with the surface antigen to such an extent that phage was not absorbed. Again, it may be observed that normal or immune serum alone, in the dilution used, has no effect on the phage. In tubes 1 and 4, where large numbers of organisms were present, the agglutinating effect of the serum was marked. In the 1:16 dilution of bacteria the clumps of organisms were very small, consisting of two or three pairs of cocci, but the great majority of bacteria were not agglutinated. Agglutination was doubtful in tube 10 (dilution 1:64), and in the dilutions of 1:128, and 1:512 there were 10–12 organisms per high power field with no clumps observed. These observations were made after one hours incubation at 37°C.

The results shown in table 4 may be explained as follows: (1) either the antibodies absorbed to the bacterial surface make any contact impossible between the bacteriophage and the bacterial phage receptors, in other words phage absorption is blocked, or (2) the aggregation of organisms in clumps so reduces the chance of contact between phage particles and bacteria that very little is absorbed, leaving a large amount free to produce plaques when placed in contact with susceptible bacteria.

We have given considerable attention to the possible rôle of agglutination as being responsible for our results, and after several experiments were done in which organisms agglutinated by lanthanum chloride were found to absorb phage as completely

as the unagglutinated controls, we feel certain that the reduced phage-absorbing capacity of suspensions of sensitized heat-killed organisms is due to a "coating" of surface antigen. Adequate evidence that our results are independent of mere mechanical clumping is shown in subsequent tables, where almost complete absorption of bacteriophage occurs in the presence of marked agglutination.

Experiment 3. The effect of varying dilutions of antiserum on the phage-absorbing power of a constant quantity of organisms

The suspending fluid was a peptone hypotonic saline solution, containing 0.1 per cent peptone (Parke-Davis) and 0.1 per cent NaCl. The solution was adjusted to pH 7.6 and sterilized in the autoclave. A heat-killed standard suspension of culture Z-1, diluted 1:32 was distributed in tubes and to these were added serial dilutions of homologous immune and normal rabbit serum, making a final dilution of organisms 1:64, and the indicated dilutions of serum. The tubes were incubated at 37°C. for 3 hours, and kept in the ice box overnight before the phage was added. An additional incubation for 20 hours at 35°C. was given, for the phage to come in contact with bacterial surface, before portions were removed from each tube and layered for plaque counts. Examinations for agglutination were made after the first incubation and again just before the phage was added. The results are listed in table 5.

One may observe in table 5 that homologous antibody in a dilution of at least 1:80 is able to coat efficiently the surface antigen so that when phage is added to this sensitized cell suspension a significant number of phage corpuscles are left free and unabsorbed. Bacterial cells treated with normal rabbit serum, on the contrary, are able to absorb the added phage completely. One may also note the lack of parallelism between the amount of agglutination and the number of phage corpuscles left unabsorbed.

The experiments described so far have been concerned with the effect of immune serum on the phage-absorbing power of suspensions of homologous organisms. The following experi-

ments deal with homologous and heterologous organisms and sera. Standard suspensions of cultures of Z-1, Z-11, and E-16 were prepared in Savita broth and killed by heating at 70°C. for 30 minutes. These standards were kept sealed in ampoules in the ice-box and appropriate dilutions made as needed, after washing once with broth. Sterility controls were always done. The organisms in these standard suspensions have retained their Gram-positiveness for the duration of this study, with little if any evidence of autolysis.

TABLE 5

TUBE NUMBER	SUSPENSION (HEAT-KILLED Z-1) 4 BILLION PER CC.	SERUM DILUTION	AGGLUTINATION		PLAQUE COUNT
			3 hrs.	18 hrs.	
1	Diluted 1:64	IS 84, 1:40	±	±	217
2	Diluted 1:64	IS 84, 1:80	±	2+	203
3	Diluted 1:64	IS 84, 1:640	2+	3+	10
4	Diluted 1:64	IS 84, 1:2560	2+	2+	4
5	Diluted 1:64	IS 84, 1:5000	±	±	2
6	None	IS 84, 1:40			230
7	None	IS 84, 1:640			304
8	Diluted 1:64	NRS, 1:40	1+	1+	2
9	Diluted 1:64	NRS, 1:80	±	2+	0
10	Diluted 1:64	NRS, 1:160	±	±	0
11	None	NRS, 1:40			230
12	None	NRS, 1:80			297
13	Diluted 1:64	None			0
14	None	Peptone-saline. None			310

IS = immune serum; NRS = normal rabbit serum.
Each tube contained a constant quantity of bacteriophage.

Experiment 4. The effect of various dilutions of homologous and heterologous sera upon the phage-binding power of a standard suspension of culture Z-11

A constant dilution of this standard suspension was used throughout; all other details with regard to technique follow those described in the previous experiments. The results are set forth in table 6.

This experiment was repeated with standard suspensions of cultures E-16 and Z-1. The results are summarized in table 7.

Here the figures represent the highest dilution of serum in the presence of which 75 per cent or more of the control number of

TABLE 6

TUBE NUMBER	SUSPENSION (HEAT KILLED) CULTURE Z-11	SERUM DILUTION (PLUS BP.)	AGGLUTINATION 20 HOURS	PLAQUE COUNT
1	Dilution 1:64	IS 11, 1:10	4+	300
2	Dilution 1:64	IS 11, 1:40	3+	297
3	Dilution 1:64	IS 11, 1:80	3+	143
4	Dilution 1:64	IS 11, 1:320	2+	30
5	Dilution 1:64	IS 11, 1:640	1+	12
6	Dilution 1:64	IS 11, 1:1280	1+	11
7	Dilution 1:64	IS 11, 1:2560	1+	10
8	Dilution 1:64	IS 87, 1:10	2+	300
9	Dilution 1:64	IS 87, 1:40	2+	300
10	Dilution 1:64	IS 87, 1:80	3+	300
11	Dilution 1:64	IS 87, 1:320	3+	197
12	Dilution 1:64	IS 87, 1:640	2+	137
13	Dilution 1:64	IS 87, 1:1280	1+	41
14	Dilution 1:64	IS 87, 1:2560	1+	15
15	Dilution 1:64	IS 84, 1:10	±	140 (cont)
16	Dilution 1:64	IS 84, 1:40	1+	230
17	Dilution 1:64	IS 84, 1:80	2+	141
18	Dilution 1:64	IS 84, 1:320	1+	16
19	Dilution 1:64	IS 84, 1:640	1+	6
20	Dilution 1:64	IS 84, 1:1280	1+	9
21	Dilution 1:64	IS 84, 1:2560	1+	7
22	Dilution 1:64	None		0
23	None	Savita broth		300

IS = immune serum; cont = contaminated.

This series also included control tubes containing serial dilutions of each serum without organisms. In each instance the numbers of plaques obtained approximated the controls.

TABLE 7

The blocking effect of immune serum on homologous and heterologous organisms

STANDARD SUSPENSION DILUTED 1:64	SERUM 84 (Z-1)	SERUM 11 (Z-11)	SERUM 87 (E-16)
Z-1	40	40	10
Z-11	40	40	80
E-16	10	40	80

phage corpuscles failed to be absorbed by the diluted standard suspension.

Upon comparison with table 1, these figures show that, as one would expect, the ability of a serum to coat the surface of an organism, thereby interfering with the absorption of phage, is in keeping with its antibody content for that organism. It is striking that although the maximum agglutinating titer of each serum is far higher, the titer for adequate blockade in the presence of a 1:64 dilution of the standard suspensions that were used is in most cases 1:40, or 1:80. We would expect that with a more dilute suspension of bacteria, a higher dilution of serum should be adequate to coat the surface antigens and therefore prevent the absorption of bacteriophage. The figures shown in table 8, where the organism suspension consisted of a standard suspension of culture E-16 diluted 1:256 confirms this.

TABLE 8

STANDARD SUSPENSION DILUTED 1:256	SERUM 84 (Z-1)	SERUM 11 (Z-11)	SERUM 87 (E-16)
Culture E-16	320	80	1280

As in table 7, the figures represent the highest titer of serum in the presence of which 75 per cent or more of the control number of phage corpuscles (as represented by plaque count) are left unabsorbed by the suspension. The difference between the sera is considerably greater in this case and the result with serum 11 is quite out of keeping with what one would expect, as the serological reactions of these organisms as given in table 1 show culture Z-11 to be more closely related to culture E-16 than to culture Z-1. However, one may observe that with a higher dilution of bacteria a considerably less amount of homologous antiserum efficiently coats the surface antigen and renders it non-susceptible to phage.

Experiment 5. The phage-blocking effect of serum from which the minor agglutinins have been removed by absorption

Standard suspensions of heat-killed cultures of Z-1 and Z-11 diluted 1:128 were placed in contact with immune serum 11 (homologous for culture Z-11), from which the group agglutinins

for culture Z-1 had been removed by absorption. The incubation periods, the examinations for agglutination, and the platings for plaque counts were made as in the previous experiments. The results are presented in table 9.

Reference to tables 1a and 7 respectively, shows that unabsorbed serum 11 agglutinates cultures Z-1 and Z-11 to equal

TABLE 9

TUBE NUMBER	HEAT-KILLED SUSPENSION	SERUM (ABSD.) DILUTION (BP. ADDED)	AGGLUTINATION		PLAQUE COUNT
			3 hrs.	20 hrs.	
1	cult. Z-1, 1:128	IS 11, 1:40	–	–	0
2	cult. Z-1, 1:128	IS 11, 1:80	–	–	2
3	cult. Z-1, 1:128	IS 11, 1:160	–	–	2
4	cult. Z-1, 1:128	IS 11, 1:320	–	–	1
5	cult. Z-1, 1:128	IS 11, 1:640	–	–	0
6	cult. Z-1, 1:128	IS 11, 1:1280	–	–	0
7	cult. Z-1, 1:128	IS 11, 1:2560	–	–	2
8	cult. Z-1, 1:128	IS 11, 1:5120	–	–	1
9	cult. Z-11, 1:128	IS 11, 1:40	–	1+	257
10	cult. Z-11, 1:128	IS 11, 1:80	–	2+	116
11	cult. Z-11, 1:128	IS 11, 1:160	–	3+	61
12	cult. Z-11, 1:128	IS 11, 1:320	–	3+	57
13	cult. Z-11, 1:128	IS 11, 1:640	–	3+	20
14	cult. Z-11, 1:128	IS 11, 1:1280	–	2+	12
15	cult. Z-11, 1:128	IS 11, 1:2560	–	2+	8
16	cult. Z-11, 1:128	IS 11, 1:5120	–	1+	6
17	None	IS 11, 1:40			300
18	None	IS 11, 1:80			300
19	None	IS 11, 1:160			300
20	cult. Z-1, 1:128	None			1
21	cult. Z-11, 1:128	None			0
22	None	Savita broth. None			300

titer and that this serum is as effective in blocking phage absorption by culture Z-1 as by the homologous organism. The present experiment demonstrates that the removal of group antibodies for culture Z-1 from this serum makes it powerless to affect phage absorption by culture Z-1. The same serum, however, still retains its ability to combine with the homologous organism, Z-11, and prevents phage absorption. Again one may

note the lack of parallelism between the degree of agglutination and phage blockade.

According to Burnet (1934) and Rakieten *et al* (1936), when watery extracts of organisms which have a phage-inactivating ability ("PIA") are placed in association with homologous immune serum and the resulting precipitate removed, the supernatants no longer possess this phage-inactivating ability. In the following experiment we investigated the phage-absorbing power of such a watery extract prepared from one of the strains of enterococci before and after the extract had been placed in contact with immune serum.

Experiment 6. The effect of immune serum upon the phage-absorbing capacity of a watery extract of enterococcus

A watery extract from culture Z-1 was made and placed in contact with immune serum 84. An initial dilution of serum of 1:5 was prepared in saline. One cubic centimeter of extract was added to one cubic centimeter of each serum dilution, incubated at 44°C. for one hour, and kept in the ice chest overnight. A definite prozone occurred in the higher concentrations of serum, and in a dilution of 1:80 a visible precipitate resulted. Each tube was centrifuged for 15 minutes at high speed and 1.75 cc. removed without disturbing any of the sediment. These constituted the so-called "supernatants." The same amount of saline was then added to each of the so-called "sediments." To all of the tubes in the series 0.05 cc. of diluted bacteriophage was added and after incubation for 22 hours at 35°C., 0.02 cc. from each tube was layered over susceptible culture Z-1. The plates were examined after the usual 20-hour incubation period and plaques counted. Table 10 summarizes the results.

The plaque counts obtained from tubes 1 to 8 were estimated, all the others were counted. It may be observed from an inspection of the results listed in table 10 that dilutions of serum up to 1:80 are able to remove all the "PIA" from the supernatant and at the same time so coat the precipitinogen with antibody that no absorption of phage takes place. In dilutions of serum above 1:80 the sediments absorb more and more phage, i.e.,

are less completely blocked. These dilutions of serum have been used repeatedly and have never in themselves, exerted any demonstrable effect on the phage. The findings with this and other extracts correspond with those obtained when suspensions of organisms were used.

During the course of this investigation, in several experiments distilled water was used as the medium, but we found that marked reduction in plaque count occurred, even in the absence

TABLE 10

TUBE NUMBER	WATERY EXTRACT	SERUM DILUTION	SUPERNATANT OR SEDIMENT (PLUS BP.)	PLAQUE COUNT
1	culture Z-1	IS 84, 1:10	Supernatant	300
2	culture Z-1	IS 84, 1:10	Sediment	300
3	culture Z-1	IS 84, 1:20	Supernatant	300
4	culture Z-1	IS 84, 1:20	Sediment	300
5	culture Z-1	IS 84, 1:40	Supernatant	300
6	culture Z-1	IS 84, 1:40	Sediment	300
7	culture Z-1	IS 84, 1:80	Supernatant	300
8	culture Z-1	IS 84, 1:80	Sediment	69
9	culture Z-1	IS 84, 1:160	Supernatant	145
10	culture Z-1	IS 84, 1:160	Sediment	87
11	culture Z-1	IS 84, 1:320	Supernatant	184
12	culture Z-1	IS 84, 1:320	Sediment	51
13	culture Z-1	IS 84, 1:640	Supernatant	118
14	culture Z-1	IS 84, 1:640	Sediment	31
15	culture Z-1	IS 84, 1:1280	Supernatant	182
16	culture Z-1	IS 84, 1:1280	Sediment	60
17	culture Z-1	IS 84, 1:2560	Supernatant	71
18	culture Z-1	IS 84, 1:2560	Sediment	12
19	culture Z-1	None	Supernatant	8
20	culture Z-1	None	Sediment	0
21	None	Saline. None	None	300

of bacteria; also that serum diluted in distilled water had an equally deleterious effect. Dilutions of the same serum in Savita broth had no effect on the same phage. In other experiments physiological saline was used as the diluent, and in these instances an even greater destruction of phage was observed. However, the addition of a small quantity of broth or serum (diluted 1:160) "protected" the phage and little or no diminution was observed. These findings appear to be at variance with the

reported experience of Rakieten, Rakieten, and Doff (1936) who state that "... bacteriophage controls were undiminished in titer for as long as 11 days in distilled water, hypertonic or hypotonic salt solution." But under the conditions of their experiments the phages were prepared in Savita broth, and this was added to tubes of distilled water or saline. Hence, a sufficient amount of "protective substance" was present. In the present study the initial dilution was prepared by adding a small loopful (less than 2 mm. in diameter) of phage to 10 cc. of saline. Further dilutions were then made by adding 0.1 cc. of this phage to tubes containing organisms suspended in saline or distilled water, with the result that not enough of the broth was carried over and the phage was destroyed. Reports on the toxicity of saline for other viruses have been made by Nicolau and Galloway (1930) and Goodpasture and Buddingh (1936).

Finally we observed that susceptible organisms suspended in distilled water had little phage-absorbing power. However, the addition of a small amount of peptone (0.1 per cent), and a small amount of electrolyte (0.1 per cent NaCl) to such a suspension in distilled water restored the phage-binding power of the suspension.

DISCUSSION

It has been shown by means of properly adjusted quantities of bacterial cells and immune serum, that the bacterial surface may be coated to such an extent that phage absorption is prevented. On the basis of the results obtained it is believed that the portion of the bacterial cell that specifically absorbs phage is the same as that which absorbs antibody from specific antisera. Since all of the organisms that we have investigated were heat-killed suspensions, it appears that the part of the cell that is primarily responsible for the absorption is the heat-stable agglutinogen. These experiments, however, do not allow us to draw any conclusions with regard to the chemical nature of the antigens involved.

In accord with the reports of Burnet (1934) and Rakieten *et al* (1936) on watery extracts of bacteria, we have found that

proper concentrations of immune serum are capable of removing all phage-inhibiting power from watery extracts of enterococci. In addition, the precipitate that is produced under conditions where precipitin and precipitinogen are present in optimal proportions, is likewise devoid of phage-inhibiting power. The more direct method of determining the fraction of the cell to which phage attaches itself, chemical analysis, has been tried (Gough and Burnet, 1934) but has not been entirely satisfactory, probably because of marked alterations in the antigens which occur when bacteria are subjected to treatment with chemicals.

CONCLUSIONS

1. The surface component of bacteria to which phage is attached preliminary to lysis can be specifically coated by anti-bacterial immune serum, and by this method rendered inaccessible to phage.

2. The heat-stable agglutinogen of the organisms studied is the surface antigen to which bacteriophage is absorbed.

3. Distilled water or saline alone, under certain conditions, appears to be toxic for the bacteriophage that we have studied.

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PROCEEDINGS OF LOCAL BRANCHES OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

CONNECTICUT VALLEY BRANCH

YALE MEDICAL SCHOOL, NEW HAVEN, Dec. 3, 1938

PRELIMINARY REPORT OF SEROLOGICAL AND CULTURAL STUDIES ON CLOSTRIDIUM HISTOLYTICUM. *George Valley and Catherine S. Flynn*, Yale University and Vassar College.

Results obtained with forty-two strains of *Clostridium histolyticum* show that surface growth on an agar medium may appear either as smooth, raised, opaque, or as irregularly branched, almost transparent colonies with slightly raised (conical) centers. A thin film may also develop. Subsurface growth in deep semisolid cysteine agar shake tubes shows small lenticular colonies in early stages; later additional units develop to form irregularly shaped coral-like colonies. Spherical to oval colonies with roughened surfaces are also observed, surrounded by a halo of very small satellite colonies. The woolly or "cotton-ball" colony frequently develops with some strains. Many of the strains yield consistently only one type of colony. It is possible that motility may play the important rôle in this phenomenon. In 1.5% agar practically all of the strains show the smooth-surfaced, irregular, coral-like colony.

The strains seem to be uniformly proteolytic and non-saccharolytic. Three or four cultures of the total of forty-two strains ferment a limited number of carbohydrates, producing both acid and gas.

Twenty antisera were prepared by injection of formolized antigens into rabbits. Agglutination tests were run with each antiserum using both formolized ("H") and phenol-alcoholized ("O") antigens. With most of the strains both "H" and "O" antigens were agglutinated to high titer (1:2560-1:5120) by the majority of the antisera; with 13.6% of the strains only the formolized antigens were agglutinated; with 4.5% of the strains neither the "H" nor the "O" antigen was agglutinated; with an additional 4.5% of strains there was agglutination of the two antigens only with an occasional serum; two of the twenty antisera failed to agglutinate the majority of the antigens, either "H" or "O".

A STUDY OF RAT PNEUMONIA. *David Borden and Walter L. Kulp*, Laboratory of Bacteriology, Connecticut State College, Storrs, Connecticut.

A small Gram-negative motile bacillus has been isolated from the lungs and heart blood of rats in the acute stage of pneumonia. This organism appears to be identical with *Bacillus bronchisepticus* both morphologically and biochemically. Serologically it has been determined that antigenic differences exist between the organism isolated from infected rats and known strains of *Bacillus bronchisepticus*. The organism in question also shares

certain antigenic components with an organism recently isolated by Eldering and Kendrick, and temporarily named by them *Bacillus para-pertussis*. Success has been obtained in 100 percent of the attempts to induce rat pneumonia by the intratracheal method of inoculation. The histopathology of the lungs of artificially infected rats appears to be identical with those of spontaneous cases. It has been found that the rat pneumonia organism, *Bacillus bronchisepticus* and *Bacillus para-pertussis* each produce a substance which is capable of oxidizing di-methyl paraphenylene diamine hydrochloride.

METHODS FOR THE DIAGNOSIS AND CONTROL OF BOVINE MASTITIS. *L. W. Slanetz and J. Naghski*, New Hampshire Agricultural Experiment Station, Durham, N. H.

Study of different tests for the diagnosis of bovine mastitis showed that there is considerable variation in the efficiency of these tests, and that their limitations must be recognized when interpreting the reactions. The strip cup, brom-thymol-blue and Hotis tests can not be relied upon for an accurate diagnosis of mastitis infection. A combination of the microscopic test, Edwards' medium and blood agar plate was very satisfactory for the detection of infected animals. It was found that the presence of leucocytes can be determined from smears of incubated samples used for the microscopic detection of streptococci. It was also demonstrated that composite samples may be employed for the diagnosis of bovine mastitis. This greatly reduces the labor, time, and materials necessary for the collection and examination of the milk samples. A plan was presented for the diagnosis and segregation of animals in a herd.

SOME FACTORS AFFECTING THE AGGLUTINABILITY OF STREPTOCOCCI ASSOCIATED WITH BOVINE MASTITIS. *W. N. Plastridge, L. F. Williams and Laura Banfield*, Department of Animal Diseases, Storrs Agricultural Experiment Station, Storrs, Connecticut.

All of the ingredients, especially the kind of peptone used in preparing nutrient broth were found to have a pronounced influence on agglutinability of udder streptococci. The percentage of usable antigens obtained from cultures grown in broths containing 0.2 percent glucose and prepared with Witte's peptone, Difco Bacto-Peptone, Armour's peptone, Difco Bacto-Tryptone, Difco Neopeptone, Fairchild's peptone and Difco Proteose-Peptone varied from 7.1 to 88.2 percent. Difco Proteose-Peptone yielded the highest percentage of usable antigens. Fresh beef infusion was decidedly superior to Savita yeast extract and commercial beef extract (Lemco). Use of phosphate buffer aided in the production of usable antigens. The optimum concentration of glucose was 0.5 percent, of sodium chloride 0.5 percent and of Difco Proteose-Peptone 8.0 percent. The optimum initial hydrogen ion concentration of buffered beef infusion broth prepared with Difco Proteose-Peptone and containing 0.5 percent glucose was pH 7.5. The optimum period of incubation was 48 hours.

Mechanical shaking and extraction with 0.5 percent "Antiformin" partly improved spontaneously agglutinable antigens. Distinctly inagglutinable or spontaneously agglutinable cells were seldom made specifically agglutinable by similar treatment.

WASHINGTON BRANCH

ARMY MEDICAL SCHOOL, WASHINGTON, D. C., DECEMBER 20, 1938

THE SAPROPHYTIC ACID-FAST BACTERIA. *Ruth Gordon*, Bureau of Plant Industry.

METHODS FOR THE INVESTIGATION OF FOOD POISONING OUTBREAKS OF BACTERIAL ORIGIN. Symposium conducted by *O. B. Williams*, National Canners Association, assisted by *A. C. Hunter and G. L. Slocum*, Food and Drug Administration; *L. H. James*, University of Maryland; *S. E. Stuart and B. L. Levine*, National Institute of Health.

ARMY MEDICAL SCHOOL, WASHINGTON, D. C., JANUARY 17, 1939

PSEUDOMONAS AERUGINOSA IN BOVINE MASTITIS. *J. F. Cone*, Division of Market-Milk Investigations, Bureau of Dairy Industry, U. S. Department of Agriculture.

Pseudomonas aeruginosa was apparently the sole etiological agent in two outbreaks of extremely severe mastitis. No mixed infections were found and most of the cows involved had had no previous history of mastitis. The acute febrile stage usually lasted only a few days, but with some of the cows the organisms were shed into the milk long after the secretion appeared normal macroscopically.

Eighteen strains of the organisms from mastitis and four strains of *P. aeruginosa* obtained from other laboratories were studied in pure cultures and were found to differ from Bergey's description of the species in their inability to produce indole, and in their ability to produce acid from some of the simpler carbohydrates.

TECHNIQUE AND RESULTS OF THE TREATMENT OF BACTERIA WITH MONOCHROMATIC ULTRAVIOLET RADIATION. *A. Hollaender*, National Institute of Health.